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DI SIENA
1240

Dipartimento di Scienze mediche, chirurgiche e neuroscienze

Dottorato in Medicina traslazionale e di Precisione

38° Ciclo

Coordinatore: Prof. Francesco Dotta

Circulating Transcriptomic signatures uncover persistent thyroid hormone signaling dysfunction in Levothyroxine-treated thyroidectomized patients

Settore scientifico disciplinare: *MEDS-08/A*

Candidata

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Firma digitale della candidata

UNIVERSITÀ DI SIENA 1240

Firmato digitalmente da Antonia Salvemini
Data: 09.03.2026 16:24:30 CET

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Anno accademico di conseguimento del titolo di Dottore di ricerca
2025/2026

Università degli Studi di Siena
Dottorato in Medicina Traslazionale e di Precisione
38° Ciclo

Data dell'esame finale
18 Marzo 2026

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Abstract

Background: Levothyroxine (LT4) monotherapy is the standard replacement therapy following a total thyroidectomy. This is based on the assumption that the conversion of thyroxine (T4) to triiodothyronine (T3) by peripheral type II deiodinase (DIO2) ensures adequate intracellular thyroid hormone (TH) signalling. However, a significant proportion of patients treated with LT4 following thyroidectomy report persistent hypothyroid-like symptoms despite biochemical euthyroidism. Previous proteomic studies from our group have demonstrated inflammatory and thrombo-inflammatory alterations, particularly in patients with reduced postoperative FT3 levels, suggesting incomplete restoration of tissue-level TH action. It is not known whether these downstream proteomic signatures reflect coordinated upstream transcriptional changes.

Methods: This prospective study involved performing paired pre- and postoperative circulating mRNA transcriptome profiling in 12 patients with biochemically euthyroidism undergoing total thyroidectomy for benign nodular disease. Plasma mRNA expression was quantified using the *NanoString nCounter® Human Metabolic Pathways Panel*. The patients were also genotyped for the DIO2 Thr92Ala polymorphism and completed longitudinal SF-36 questionnaires. We used differential gene expression (DEG), gene set enrichment analysis (GSEA) and unsupervised clustering of quality-of-life (QoL) scores to integrate clinical, genetic and molecular data.

Results: Paired analysis revealed postoperative upregulation of genes involved in extracellular matrix remodelling (*THBS1* and *ITGB1*), inflammatory and chemokine signalling (*CCL5* and *CTSA*), lipid metabolism (*PTGS1*) and modulation of the PI3K/AKT pathway. DIO2 Thr92Ala carriers displayed a distinct transcriptional phenotype characterised by the downregulation of mitochondrial detoxification (*ALDH2*), stress-response kinases (*MAPK8*) and ECM components (*LAMB1*), as well as the upregulation of metabolic regulators (*RPS6KB1* and

NDUFB4). Unsupervised clustering of SF-36 scores identified two quality of life (QoL) subgroups: patients with poorer postoperative QoL exhibited transcriptional signatures enriched for oxidative stress, neuro-immune activation (*CTSS* and *GLUL*) and astrocyte-metabolic pathways.

Conclusions: This study provides the first evidence from paired human circulating transcriptomes that levothyroxine monotherapy does not consistently restore tissue-level euthyroidism after total thyroidectomy, even when serum TSH levels are normalised. The postoperative mRNA landscape revealed sustained activation of inflammatory, metabolic and extracellular matrix (ECM)-integrin remodelling pathways, which were further modulated by DIO2 genotype and patient-reported symptom burden. These findings highlight the limitations of monitoring only biochemical markers and provide strong evidence in favour of integrating transcripts-based biomarkers to identify patients at risk of persistent dysfunction. This would enable more accurate stratification of postoperative vulnerability and guide truly precision-tailored thyroid hormone replacement therapy.

1. Introduction

1.1 Thyroid Gland

The Thyroid gland is an endocrine gland situated in the lower front of the neck. It is responsible for synthesizing and secreting the thyroid hormones thyroxine (T4) and triiodothyronine (T3). These hormones exert widespread control over energy metabolism, thermogenesis, cardiovascular function, somatic growth and the development and maintenance of the central nervous system. A smaller population of cells known as C cells, which are located within the gland, produce Calcitonin, a hormone that participates in calcium homeostasis [1].

In adults, therefore, the thyroid represents a central regulatory node that integrates neural inputs, metabolic demands and the function of multiple peripheral organs, including the cardiovascular system, bones, skeletal muscles, the haematopoietic system and the reproductive system.

1.1.1 Anatomy and Physiology of the Thyroid gland

The Thyroid gland is a highly vascularized endocrine organ located in the front of the neck. It typically spans the C5-T1 vertebral levels, consisting of two lateral lobes that are connected by an isthmus. The gland receives its arterial supply primarily from the superior thyroid arteries (branches of the external carotid artery) and the inferior thyroid arteries (from the thyrocervical trunk), while venous drainage occurs through the superior, middle and inferior thyroid veins. Innervation is predominantly autonomic, with sympathetic fibres from the cervical ganglia and parasympathetic fibres from the vagus nerve modulating vascular tone rather than hormone secretion. Histologically, the gland comprises spherical follicles whose epithelial cells synthesize and secrete the iodothyronines T4 and T3. Meanwhile, the parafollicular C cells, which are derived from the ultimobranchial body, produce calcitonin [2] (Figure 1).

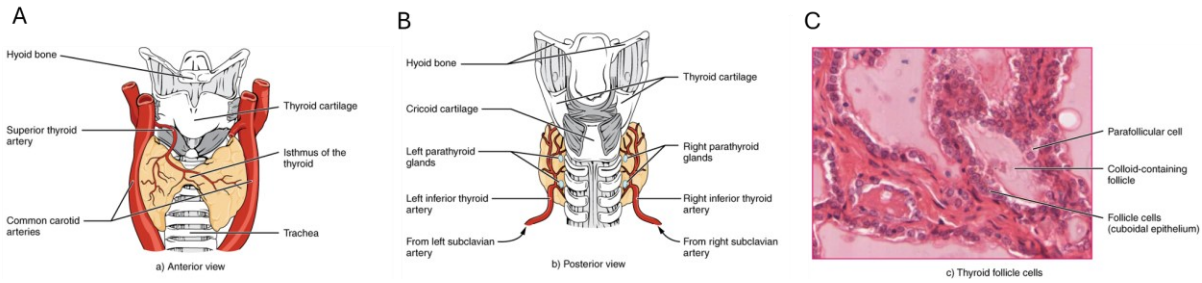

Figure 1. *(A) Anterior view showing the thyroid lobes, isthmus, and major arterial supply. (B) Posterior view highlighting parathyroid gland position and inferior thyroid artery branches. (C) Histological section of thyroid tissue illustrating follicles lined by cuboidal follicular cells and interspersed parafollicular (C) cells.*

1.1.2 Thyroid Hormone Synthesis

Thyroid hormone biosynthesis occurs within the follicular architecture of the thyroid gland through a sequence of tightly regulated steps, initiated by thyroid stimulating hormone (TSH) stimulation [3] (Figure 2). This process starts with the production of thyroglobulin (Tg), a large iodinated glycoprotein formed in the rough endoplasmic reticulum of thyrocytes. It is then processed in the Golgi apparatus and released into the follicular lumen, where it acts as a framework for TH assembly. In parallel, iodide is actively concentrated from the bloodstream into thyrocytes via the basolateral sodium-iodide symporter (NIS), driven by the Na^+/K^+-ATPase pump. Iodide then diffuses the apical membrane through pendrin (SLC26A4) and related transporters to enter the colloid space. At the apical pole, thyroid peroxidase (TPO), supported by hydrogen peroxide generated by dual oxidase (DUOX), catalyses the sequential oxidation of iodide to iodine and its organification onto the tyrosyl residues of Tg, as well as the coupling of monoiodotyrosine (MIT) and diiodotyrosine (DIT) to form triiodothyronine (T3) and thyroxine (T4).

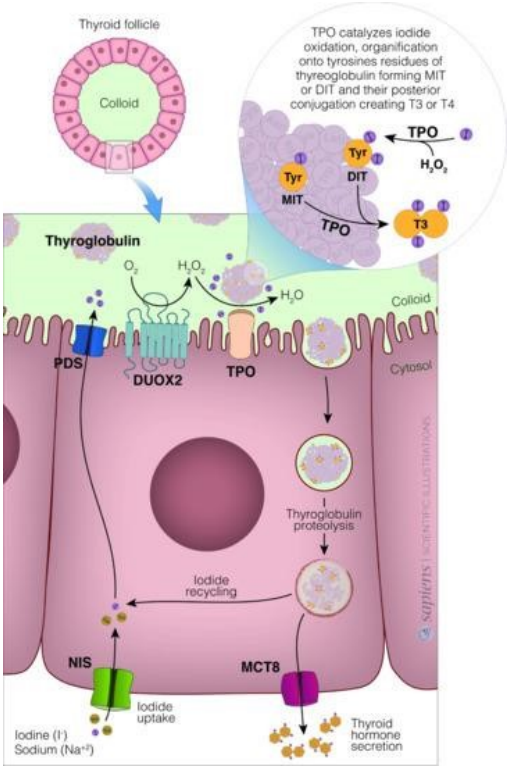

Figure 2. *Schematic representation of thyroid hormone biosynthesis. Iodide uptake (NIS, PDS), oxidation and organification by TPO/DUOX2, and coupling on thyroglobulin generate MIT/DIT and ultimately T3/T4. Subsequent endocytosis and lysosomal proteolysis allow hormone release and intracellular iodide recycling.*

The Tg stored in the colloid, constitutes the principal intrathyroidal reservoir of hormone precursors. Upon TSH stimulation, Tg undergoes endocytosis at the apical membrane and is directed to endolysosomal compartments. There, proteolysis liberates T4 and T3 for secretion into the circulation. The MIT and DIT released during Tg degradation are deiodinated by iodotyrosine dehalogenase (DEHAL1), which enables efficient intrathyroidal iodine recycling [4,5]. Overall, approximately 80% of the released hormone is T4 and 20% is T3, reflecting the intrinsic coupling efficiency of TPO [6]. The synthesis and release of T4 and T3 are the primary endocrine outputs of the thyroid gland and are essential for the function of the hypothalamic-pituitary-thyroid (HPT) axis.

1.1.3 Transport and Mechanisms of Action of Thyroid Hormones

The cellular entry of thyroid hormones is mediated by a set of specialized plasma membrane transporters rather than passive diffusion. The best characterized systems enabling the influx and efflux of T4 and T3 into target cells, are monocarboxylate transporters MCT8 and MCT10, the organic anion-transporting polypeptide OATP1C1, SLC17A4, and the L-type amino acid transporters LAT1 and LAT2 [7]. Their tissue-specific expression patterns, regulate intracellular hormone availability.

Within target cells, T3 primarily acts through nuclear TH receptors TRα and TRβ, which are ligand-dependent transcription factors encoded by *THRA* and *THRB* [8]. These receptors bind thyroid hormone response elements (TREs) as homo- or heterodimers with RXR, thereby regulating the transcription of primary T3-responsive genes and modulating broader gene networks via secondary transcription factors and non-coding RNAs [9].Binding of the ligand, promotes the recruitment of coactivator complexes with histone acetyltransferase activity. In contrast, unliganded receptors maintain chromatin in a repressed state through corepressor complexes [10]. The differential expression of TR isoforms is responsible for tissue-specific sensitivity to thyroid hormones, and explains the phenotypic variability observed in receptor mutations [11](Figure 3).

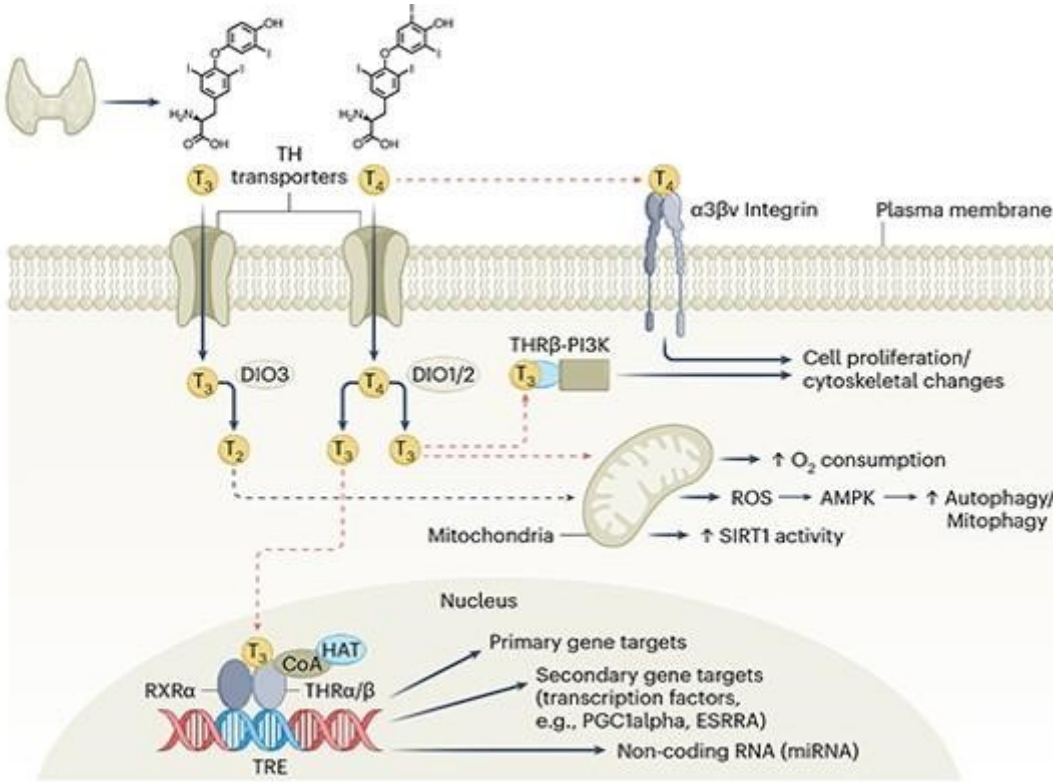

Figure 3. *Overview of the genomic and non-genomic actions of thyroid hormones. T4 enters cells via specific transporters and is converted to T3 by DIO1/DIO2, while DIO3 inactivates THs. T3 regulates gene expression through nuclear TRα/β receptors and also activates rapid extranuclear pathways involving mitochondria and integrins, influencing energy metabolism, ROS production, and autophagy.*

In addition to their classical genomic actions, thyroid hormones initiate rapid non-genomic signalling through extranuclear pathways [12]. These responses involve binding to cytosolic proteins, mitochondrial sites or membrane receptors, such as integrin αVβ3, which activate PI3K, MAPK and other kinase pathways, independently of transcription [13]. Non-genomic signalling contributes to the regulation of mitochondrial respiration [14], neuro-glial cytoskeleton [15], angiogenesis [16] and proliferative responses [17] (Figure 3). These actions can be elicited not

only by T3, but also by T4 and various iodothyronine metabolites, highlighting the complexity of thyroid hormone signalling across cellular compartments.

1.1.4 Regulation of Thyroid Function

Thyroid function is regulated by the hypothalamic-pituitary-thyroid (HPT) axis. In this axis, hypophysiotropic TRH neurons in the paraventricular nucleus (PVN) integrate homeostatic, metabolic, circadian and environmental inputs to control the release of TSH from the pituitary gland, ultimately regulating thyroid hormone secretion [18]. Despite representing less than 2% of total brain volume, the hypothalamus's nuclear organisation enables the precise control of energy balance, thermogenesis, feeding behaviour, reproduction and circadian rhythms, all of which converge on TRH-producing neurons [19,20]. TRH is released from PVN neurons into the median eminence and transported through the portal system to the anterior pituitary, where it binds to TRH-R1 receptors to stimulate TSH synthesis and secretion [20]. TSH then drives all major steps of thyroid hormone biosynthesis, resulting in the release of T4 (~80%) and T3 (~20%) (Figure 4).

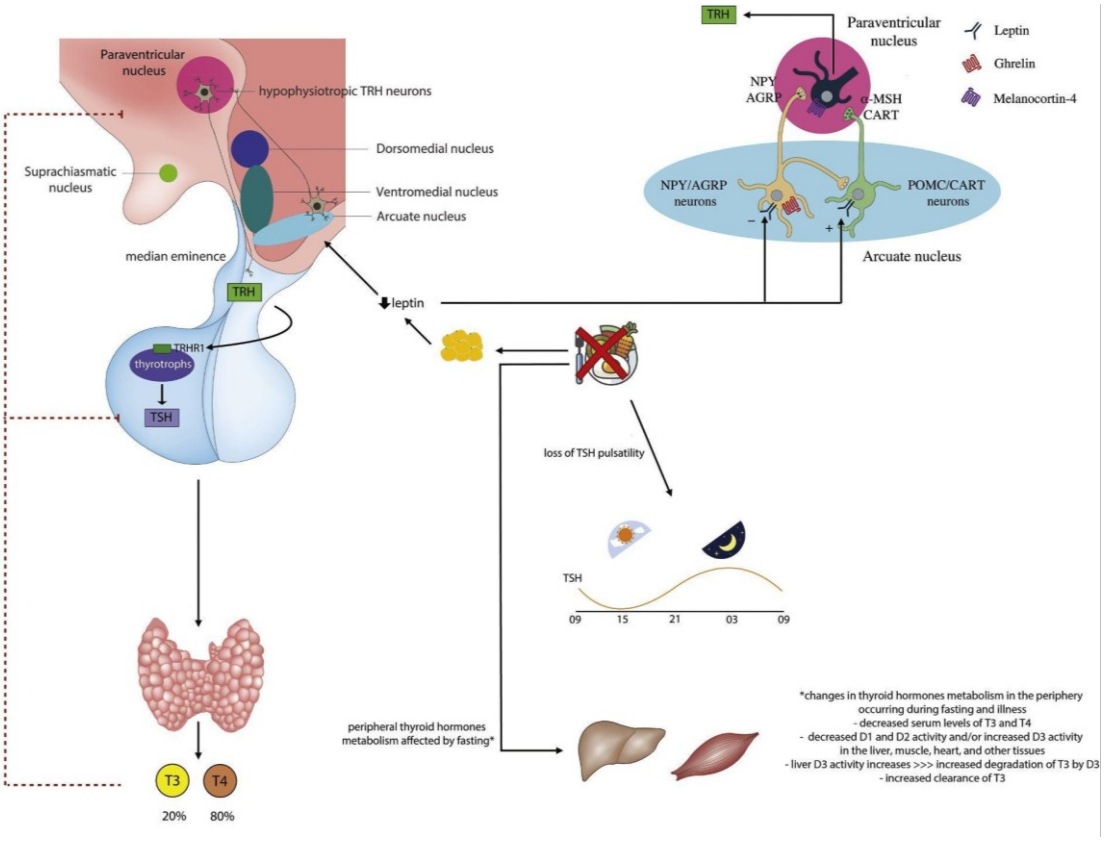

Figure 4. *Schematic overview of HPT axis regulation integrating metabolic signals. Leptin, ghrelin, and melanocortin-derived peptides modulate TRH-producing neurons in the paraventricular nucleus, influencing TSH secretion and its circadian pulsatility. During fasting or metabolic stress, reduced leptin signaling and increased peripheral DIO3 activity lower circulating T3/T4 levels, illustrating the interplay between energy status and thyroid hormone homeostasis.*

TRH neurons receive dense afferent input from arcuate nucleus POMC/CART neurons, which stimulate TRH transcription, and from NPY/AgRP neurons, which exert inhibitory actions, particularly during fasting when leptin levels decrease [20]. This bidirectional modulation dynamically adjusts the TRH transcriptional threshold, enabling the HPT axis to downregulate metabolism in response to reduced energy availability. During fasting, declining leptin levels reduce α-MSH signalling and enhance AgRP activity. Both of these factors suppress TRH

production and blunt TSH pulsatility, producing a transient central hypothyroid state despite reduced peripheral T4/T3 levels [21]. A similar reduction in TRH expression occurs during acute and chronic illness. This is driven by cytokines such as IL-1, IL-6 and TNF-α, as well as by inflammation-induced D2 upregulation in tanycytes. This produces the characteristic biochemical pattern of non-thyroidal illness syndrome [22]. Negative feedback is primarily mediated by T3, which suppresses prepro-TRH in the PVN and rapidly downregulates TSH-β and α-glycoprotein hormone subunit (αGSU) transcription [23]. Pituitary TSH secretion exhibits pulsatile release (one pulse every 1–2 hours) and circadian rhythmicity, featuring a nocturnal surge driven by the suprachiasmatic nucleus, independent of feedback regulation [24]. TRH remains the major positive regulator of TSH, while thyroid hormones exert potent inhibitory effects, both directly via nuclear TRs in thyrotropes and indirectly by suppressing TRH synthesis. Other modulators include somatostatin, dopamine, catecholamines, ghrelin, cytokines and pituitary-derived peptides, which adjust TSH secretion in response to stress, fasting, inflammation and metabolic status [22,25].

Together, these mechanisms confer remarkable stability to individual TSH set-points, which are genetically influenced and highly reproducible, while preserving the capacity for rapid adaptive modulation in response to changes in energy availability, circadian phase, illness or environmental challenges.

1.2 Thyroid Hormon action at cellular level

1.2.1 *Intracellular Availability of Thyroid Hormones*

The intracellular availability of THs reflects the interplay between circulating free thyroxine (FT4), local conversion to T3, transporter-mediated uptake, and tissue-specific metabolic demands. While FT4 is generally considered the most reliable systemic indicator of thyroid

function, intracellular T3 concentrations can differ significantly from circulating FT3 levels because each organ controls the entry, activation and inactivation of THs, differently.

Once inside the cell, T4 and T3 concentrations diverge further due to tissue-specific expression of deiodinases. DIO2 generates T3 locally from T4, thereby amplifying intracellular T3 signalling. In contrast, DIO3 terminates TH signalling by converting T4 to rT3 and T3 to T2 [26]. This creates organ-specific TH microenvironments that are largely independent of serum FT3. For example, skeletal muscle expresses high levels of DIO2, enabling substantial local T3 production to support fibre-type switching, mitochondrial biogenesis and thermogenesis [27–29]. In contrast, the liver primarily expresses DIO1, which contributes to circulating T3, but plays a limited role in hepatic intracellular T3 generation. This is because T3 signalling in hepatocytes is more tightly controlled by transporters and the abundance of nuclear THRβ [30]. Brown adipose tissue (BAT) relies almost exclusively on DIO2 to generate intracellular T3, which allows for the rapid activation of thermogenin/uncoupling protein 1(*Ucp1*), lipolysis, mitochondrial biogenesis and thermogenesis in response to sympathetic stimulation [31,32]. The CNS is particularly sensitive to the balance of transporters and deiodinases: OATP1C1 and MCT8 regulate the entry of T4 and T3, while the expression of regional DIO2 and DIO3 shapes the availability of T3 during critical developmental periods [33]. In the cerebellum, retina and cochlea, the precise timing of local TH activation is crucial for neuronal differentiation, synaptogenesis, dendritic arborisation and sensory system maturation [33]. In the heart, where THRα predominates, intracellular T3 drives contractility, calcium handling, electrical conduction and metabolic substrate switching. Here, transporter-mediated uptake predominates over deiodinase activity [34]. In bone tissue, both MCT8/MCT10 and DIO2/DIO3 regulate the local availability of thyroid hormones, enabling T3-dependent differentiation of osteoblasts, hypertrophy of chondrocytes, and remodelling of bone [35–37].

Therefore, intracellular T3 availability represents the true determinant of TH action, and its regulation is highly tissue-specific and context-dependent, only being partially reflected in circulating hormone levels.

1.2.2. *Role of Deiodinases*

Thyroid hormone deiodinases are selenoproteins that finely regulate intracellular thyroid hormone signalling. They do this, by catalyzing outer- or inner-ring deiodination of iodothyronines. This activates or inactivates T4 and T3 in a tissue- and context-specific manner [38]. All three enzymes are integral membrane proteins: *DIO1* and *DIO3* are located at the plasma membrane, while *DIO2* is positioned in the endoplasmic reticulum to deliver newly generated T3 directly to the nucleus [39,40]. These subcellular differences underpin their distinct physiological functions.

DIO1 catalyses both outer- and inner-ring deiodination of T4 and rT3 [41]. Predominantly expressed in the liver, kidneys and thyroid, DIO1 contributes substantially to circulating T3 in rodents but plays a comparatively smaller role in humans, largely acting as a scavenger for sulfated iodothyronines destined for biliary and urinary excretion. DIO1-generated T3 equilibrates rapidly with the plasma; however, little contributes to sustained intracellular T3 signalling [38].

DIO2 is the primary intracellular deiodinase responsible for converting T4 to T3 with nanomolar affinity [42]. Its short half-life of ~40 minutes, driven by substrate-induced ubiquitination and proteasomal degradation, enables the rapid modulation of T3 production within cells [43]. The deubiquitinases USP20 and USP33 can prolong the half-life of DIO2, particularly in BAT and glial cells, where DIO2 is highly responsive to cAMP and adrenergic stimulation [44]. DIO2 is highly expressed in the CNS, the pituitary gland, the cochlea, the

skeleton, BAT, the myocardium and skeletal muscle [42]. In these tissues, DIO2 provides most of the nuclear T3, with up to 50% of TR-bound T3 originating from local DIO2 activity rather than from circulating hormones [42]. DIO2 also mediates adaptive thermogenesis in BAT, where norepinephrine-driven induction of DIO2 synergises with PGC-1α and UCP1 to accelerate lipid oxidation and heat production. Furthermore, T3 generated by DIO2 supports mitochondrial biogenesis, myocyte contractility, and behavioural phenotypes related to mood and anxiety [42]. The DIO2 Thr92Ala polymorphism impairs enzyme trafficking and induces endoplasmic reticulum (ER)/Golgi stress, mitochondrial dysfunction and neuronal vulnerability [45]. Castagna et al. [46]demonstrated that the Thr92Ala polymorphism also reduces DIO2 protein stability and T4-to-T3 conversion in vivo. This leads to significantly lower postoperative FT3 levels in patients who have undergone thyroidectomy and are on Levothyroxine (LT4) therapy, compared with wild-type carriers. This increases the risk of persistent relative T3 deficiency despite biochemical euthyroidism.

DIO3 is the primary deiodinase responsible for the conversion of T4 and T3 to rT3 and T2 [47]. It is highly expressed in embryonic tissues, the placenta and select CNS regions, where it counterbalances DIO2 to shape developmental TH gradients [48]. In adults, DIO3 expression is low, but it is strongly inducible by hypoxia, ischaemia, inflammation and oxidative stress, resulting in localised tissue hypothyroidism [38].

Together with DIO1 and DIO2, DIO3 forms a tissue-specific network that defines intracellular T3 availability independently of serum levels. After thyroidectomy, reliance on extrathyroidal deiodination becomes absolute. DIO1 sustains circulating T3, whereas adequate intracellular T3, especially in the brain, heart and muscles, requires efficient DIO2 activity [38]. Variability in DIO2 expression, ubiquitination and transporter systems can lead to heterogeneous tissue T3 levels despite normal FT4 and TSH levels. This contributes to low FT3 levels and residual

symptoms under LT4 monotherapy. Impaired DIO2 function, including Thr92Ala, may further predispose individuals to hypothyroidism at the tissue level [46].

1.3 Thyroid Disorders

1.3.1 Benign Thyroid Disorders

Benign thyroid disorders arise from disturbances in hormone production, regulation or glandular architecture. The most frequent presentation is *Structural abnormalitie*s: nodular thyroid disease is palpable in 0.8–1.5% of men and 5.3–6.4% of women, and most lesions are benign colloid nodules, thyroid adenomas, cysts, or part of an unrecognised multinodular goitre. *Multinodular goitre* reflects chronic, heterogeneous follicular hyperplasia driven by iodine deficiency, environmental or dietary factors, or defects in hormone synthesis. This results in progressive development of functional autonomy.

Hyperfunctioning disorders result from the production of excess thyroid hormones. Graves' disease is mediated by thyroid-stimulating immunoglobulins that activate the TSH receptor and induce diffuse hyperthyroidism. Autonomous hormone secretion can be caused by a toxic adenoma or toxic multinodular goitre, in which hyperfunctioning nodules escape pituitary feedback control. Severe exacerbations of thyrotoxicosis may culminate in thyroid storm, which is driven by hormone-induced sensitization to catecholamines.

In contrast, *Hypofunctioning disorders* are characterized by insufficient thyroid hormone action. Primary hypothyroidism most commonly results from autoimmune thyroiditis, whereas iatrogenic hypothyroidism follows thyroidectomy or radioiodine therapy. Congenital hypothyroidism stems from gland dysgenesis or dyshormonogenesis. Thyroid hormone resistance and non-thyroidal illness syndrome, on the other hand, reflect impaired hormone action or altered peripheral metabolism rather than defective secretion.

Together, these benign disorders highlight the diverse immune, metabolic, structural and genetic mechanisms that can disrupt thyroid physiology [49].

1.3.2 Malignant Thyroid Disease

Malignant thyroid disease is a heterogeneous group of neoplasms whose global incidence has risen substantially in recent decades. This increase is partly due to improved detection, but is also due to environmental exposures such as radiation, pollutants and endocrine-disrupting chemicals. Thyroid cancer is now the most prevalent endocrine malignancy, exhibiting significant female predominance and notable geographic variation in incidence. Papillary thyroid carcinoma (PTC) accounts for almost 90% of cases and is usually caused by alterations that activate the MAPK pathway, such as BRAF, RAS, or RET/PTC rearrangements. Despite frequently involving the lymph nodes, PTC generally has an excellent prognosis. Follicular thyroid carcinoma (FTC), which is more prevalent in iodine-deficient regions, often carries RAS mutations, TERT promoter variants or PAX8-PPARG fusions, and shows a higher propensity for haematogenous spread. Medullary thyroid carcinoma (MTC) originates from the C cells of the thyroid gland and is driven by germline or somatic RET mutations. It may occur sporadically or within multiple endocrine neoplasia type 2 (MEN2) syndromes. Anaplastic thyroid cancer (ATC), which is rare, displays profound dedifferentiation, rapid progression and complex genomic disruption, including alterations to TP53, TERT, PIK3CA and CDKN2A/B, resulting in an extremely poor prognosis [50]. While this work does not focus on malignant disease, providing an overview of it is essential for distinguishing benign structural disorders, particularly multinodular goitre, from their malignant counterparts in clinical assessment.

1.4 Focus on Euthyroid Multinodular Goiter (MNG)

1.4.1 Epidemiology and Physiopathology

Multinodular goitre (MNG) is the final stage of diffuse thyroid hyperplasia, which evolves into structurally and functionally heterogeneous nodules. Its prevalence varies according to iodine intake, reaching 30–50% in autopsy and ultrasound surveys. However, clinically evident MNG is present in fewer than 5% of individuals in regions with sufficient iodine, and there is a strong female predominance [51,52]. In euthyroid MNG, nodules arise from repeated cycles of follicular hyperplasia and involution, which are driven by chronic, low-grade TSH stimulation [53–55]. Functional autonomy may progressively develop even in biochemically euthyroid glands, as indicated by impaired TRH-induced TSH responses and relatively higher T3 levels despite normal T4 [56]. Genetic predisposition, somatic TSH-R mutations and environmental factors, such as borderline iodine intake, can further amplify clonal expansion and nodular transformation [57]. Thus, euthyroid MNG is a multifactorial condition in which structural complexity coexists with apparently normal systemic thyroid hormone levels.

1.4.2 Diagnosis and Laboratory Evaluation

Clinical evaluation of euthyroid multinodular goiter, focuses primarily on detecting mass-effect symptoms, as patients often report slowly progressive cervical enlargement, tracheal deviation, dysphagia, or cough. Physical examination alone is unreliable for defining gland size or nodule number, making laboratory and imaging studies indispensable.

Biochemically, MNG is usually associated with normal TSH and FT4/FT3 levels, although mild TSH suppression may indicate early autonomy. Anti-TPO and anti-Tg Antibody testing helps exclude coexisting autoimmune thyroiditis. Serum Tg, while often elevated, lacks diagnostic specificity. TSH measurement is central also during follow-up, ensuring maintenance of euthyroidism and early detection of postoperative or progressive dysfunction.

Ultrasound is the first-line imaging evaluation, offering high sensitivity for nodule detection, volume estimation, and guidance for fine-needle aspiration biopsy (FNAB). Scintigraphy is reserved for functional assessment and identification of autonomous nodules, whereas CT or MRI is indicated for substernal goiters or suspected airway compression.. FNAB remains the diagnostic gold standard for cytologic characterization of dominant or suspicious nodules, with Ultrasound guidance significantly enhancing accuracy [58].

1.4.3 Treatment of Euthyroid Multinodular goiter

The management of MNG remains individualized due to the absence of a universally optimal therapy. Iodine supplementation is generally avoided due to the risk of iodine-induced thyrotoxicosis and the possibility of increased PTC or thyroiditis. Levothyroxine suppressive therapy only modestly and transiently reduces the size of the thyroid gland, requires sustained TSH suppression and carries risks of subclinical hyperthyroidism, such as atrial fibrillation, bone loss and cardiovascular morbidity, making it unsuitable for most patients with established MNG. Surgery is the preferred treatment for enlarging, symptomatic or substernal goiters. It provides rapid decompression, definitive histology and durable volume reduction. Permanent recurrent laryngeal nerve or parathyroid injury occurs in less than 1% of cases in high-volume centres. Recurrence after subtotal thyroidectomy occurs in 15–40% of cases, prompting many centers to adopt total thyroidectomy to eliminate the risk of regrowth. Postoperative management requires lifelong LT4 replacement therapy, titrated to maintain TSH levels within the normal range and prevent hypothyroidism. Routine TSH-suppressive therapy to prevent recurrence, is not recommended as randomised trials demonstrate minimal benefit [58].

Overall, although surgery remains the most definitive treatment for multinodular goiter, the resulting lifelong dependence on LT4 replacement therapy exposes patients to the well-

recognized limitations of monotherapy, most notably suboptimal T3 availability and residual hypothyroid-like symptoms, despite biochemical euthyroidism. This provides a compelling rationale for investigating post-thyroidectomy thyroid hormone physiology and systematically assessing patient-reported quality of life.

1.4.4 Limitations of Levothyroxine (LT4) Monotherapy

Although LT4 replacement therapy is the standard of care following a total thyroidectomy, achieving stable biochemical euthyroidism remains a clinical challenge, particularly in patients without a thyroid gland who lack endogenous T3 secretion and therefore rely entirely on peripheral T4-to-T3 conversion. Although international guidelines recommend adjusting LT4 doses to keep TSH levels within specific reference ranges, these ranges do not always reflect tissue-level euthyroidism. This can lead to a mismatch between biochemical targets and patients' perceived well-being [59]. Emerging evidence shows that, despite normalized TSH and often elevated FT4 levels, a substantial proportion of LT4-treated thyroidectomized patients exhibit lower postoperative FT3 levels (15–34%, depending on the cohort). This reflects an absence of thyroidal T3 secretion and variable deiodinase efficiency [46].

This dissociation between TSH normalization and persistent FT3 reduction is clinically relevant: approximately 20% of athyreotic patients fail to achieve physiological T3 levels unless LT4 doses suppress TSH, and a consistent subset continues to report hypothyroid-like symptoms, such as fatigue, weight gain, cognitive impairment and depression, despite 'normal' laboratory profiles [60]. Genetic determinants further compound these discrepancies: the common DIO2 Thr92Ala polymorphism, present in 13–15% of the population, has been associated with reduced D2-mediated T3 generation in vivo [61]. Our study provides direct evidence that patients carrying the Ala allele have significantly lower postoperative FT3 levels than individuals with the Thr allele, despite equivalent TSH and FT4 concentrations. The magnitude of the FT3 reduction is proportional to the number of Ala alleles present (36.5% in Thr/Ala and 58.3% in

Ala/Ala) [46]. The clinical implications are considerable. LT4 monotherapy may not fully restore euthyroidism in genetically predisposed or deiodinase-dependent tissues, particularly skeletal muscle and the pituitary gland. This mirrors findings from preclinical models, where LT4 alone failed to normalize tissue T3 content, whereas combined LT4+LT3 therapy did [62]. Although randomized trials in hypothyroid patients have not uniformly demonstrated the superiority of combination therapy in terms of symptoms [63], guidelines acknowledge that LT4+LT3 therapy may be considered for individuals with persistent symptoms despite normalized TSH levels [64]. In practice, optimizing the LT4 dose is further hindered by factors such as impaired gastrointestinal absorption, which is exacerbated by postoperative calcium supplementation or mucosal injury induced by radioiodine, as well as interindividual variability in LT4 pharmacokinetics. Although novel formulations (e.g. liquid or soft-gel LT4) and computational dose-adjustment algorithms[65] may improve stability and adherence, they do not address the underlying issue of T3 dependency in the athyreotic state.

Taken together, these findings highlight a key limitation of LT4 monotherapy: normalizing serum TSH levels does not ensure sufficient tissue T3 levels, especially in patients with impaired deiodinase activity or genetically determined reductions in D2 efficiency. This physiological mismatch provides a strong rationale for examining patient-reported outcomes and quality-of-life metrics as complementary indicators of postoperative thyroid hormone sufficiency.

1.4.5 Quality of Life Assessment in Thyroidectomy Patients

Despite biochemical euthyroidism on LT4 therapy, a substantial proportion of thyroidectomized patients report persistent hypothyroid-like symptom including fatigue, mood disturbance, cognitive slowing, sleep difficulties, and reduced well-being [66,67]. Several studies have shown that LT4-treated individuals, particularly those exposed to TSH suppression, report higher rates of depression, anxiety, sleep impairment, and decreased emotional and physical

functioning compared with both healthy controls and non-LT4-treated surgical patients [66]. These symptoms may stem from alterations in FT3 availability, inter-individual variability in deiodinase efficiency, or non-thyroidal mechanisms affecting neurocognitive and metabolic homeostasis, and they persist even when TSH and FT4 remain within reference intervals [67,68]. Given these challenges, validated Patient-Reported Outcome (PRO) measures are essential for quantifying the subjective symptom burden experienced by thyroidectomized patients. Among available instruments, the 36-Item Short Form Health Survey (SF-36) is the most widely adopted generic measure of health-related quality of life in clinical research and routine care. Developed within the *Medical Outcomes Study*, the SF-36 assesses eight core dimensions of health status: *Physical functioning, Role limitations due to physical or emotional problems, Bodily Pain, General mental health, Vitality, Social functioning*, and *General health perceptions*, along with a single item on perceived *Health change* [69,70]. Because it provides a comprehensive overview of physical, emotional, and functional well-being, the SF-36 is particularly suited to thyroid disorders, where symptoms often span multiple domains not fully captured by biochemical markers. In patients with hypo- or hyperthyroidism, the SF-36 reliably detects alterations in vitality, emotional well-being, physical performance, and pain, and it has proven sensitive to clinical transitions such as restoration of euthyroidism and changes in LT4 dosing [71].

A pre- and postsurgery longitudinal approach is essential, as it allows assessment of baseline QoL before the onset of LT4 dependence and captures the dynamic evolution of symptoms following thyroidectomy, including the impact of LT4 dose, TSH suppression strategies, and individual recovery trajectories. Such designs help distinguish surgery-related effects from hormone-related effects and provide a more accurate evaluation of patient-specific deviations from pre-surgical functional status.

Our previous work demonstrated that a subset of thyroidectomized patients, independent of DIO2 Thr92Ala genotype, exhibits reduced FT3 levels despite normal TSH, accompanied by persistent symptoms and lower QoL [46,72]. Motivated by the hypothesis that biochemical euthyroidism may mask underlying biological dysregulation, we investigated circulating proteomic profiles in patients with reduced versus stable FT3. This analysis revealed perturbations in coagulation, complement activation, and lipoprotein remodeling pathways-processes linked to systemic and endothelial inflammation, which is known to contribute to fatigue, mood symptoms, and cognitive dysfunction [73]. To strengthen these observations, we subsequently expanded the analysis by including healthy euthyroid controls, confirming that thyroidectomized patients, particularly those with reduced FT3, display a distinct inflammatory and thrombo-inflammatory proteomic signature characterized by dysregulated complement cascades, altered HDL proteoforms, and serine-protease/serpin imbalance [74]. Together, these findings support the concept that persistent symptoms in biochemically euthyroid LT4-treated patients may derive from tissue-level hypothyroidism driven by chronic low-grade inflammation and impaired proteolytic remodeling. This mechanistic framework provides a strong rationale for integrating PROs, biochemical markers, and multi-omic profiling in the evaluation of post-thyroidectomy QoL.

2. Aims of the Study

Despite achieving biochemical euthyroidism, a substantial proportion of thyroidectomized patients on levothyroxine (LT4) replacement therapy continue to experience symptoms compatible with hypothyroidism, suggesting a potential mismatch between circulating thyroid hormone levels and intracellular thyroid hormone action. Building on our earlier proteomic findings showing persistent inflammatory, vascular, and metabolic alterations in LT4-treated patients with reduced FT3 availability, this study was designed to investigate the molecular

mechanisms underlying postoperative symptom persistence in individuals undergoing total thyroidectomy for benign thyroid disease, such as MNG in euthyroidism.

1. The *primary aim* was to characterize the biological impact of thyroidectomy and subsequent LT4 replacement by performing paired pre- and post-surgery transcriptomic profiling in patients who maintained biochemical euthyroidism (TSH 0.4–4.0 mU/L) throughout follow-up. This approach aimed to determine whether surgery and LT4 monotherapy induce coherent transcriptional adaptations in circulating mRNA that are not captured by standard thyroid function tests.
2. The *secondary aim* was to assess whether genetic variability in thyroid hormone metabolism, specifically the *DIO2 Thr92Ala* polymorphism, modulates the transcriptional response to thyroidectomy and influences the molecular phenotype under LT4 therapy.
3. A *third exploratory aim* was to integrate transcriptomic data with patient-reported outcomes derived from the SF-36 questionnaire, using unsupervised clustering to identify symptom-based subgroups. This analysis aimed to determine whether patients reporting lower postoperative quality of life exhibit distinct circulating molecular signatures consistent with impaired tissue thyroid hormone action, oxidative stress, or immune activation.

Overall, the study sought to determine whether biochemical euthyroidism under LT4 monotherapy corresponds to molecular and symptomatic homeostasis, or whether multi-level biological disturbances persist in a subset of patients despite normal serum TSH and thyroid hormone concentrations. By addressing this question, the study provides a framework for identifying transcriptomic biomarkers of postoperative vulnerability and for guiding future personalized approaches to thyroid hormone replacement after total thyroidectomy.

3. Materials and Methods

3.1 Study population

This was a prospective, monocentric, no-profit study conducted at the *Endocrinology Unit of Policlinico Santa Maria alle Scotte, Siena*. A total of 27 consecutive patients, scheduled to undergo total thyroidectomy or benign or low-risk nodular thyroid disease were initially evaluated for eligibility. After applying predefined inclusion and exclusion criteria, 12 patients were deemed eligible and enrolled in the study (Table 1). Inclusion criteria were: (i) age between 18 and 70 years; (ii) diagnosis of benign nodular thyroid disease or differentiated thyroid carcinoma not requiring postoperative suppressive therapy; (iii) euthyroid state at baseline, defined as serum TSH between 0.5 mU/L and 4.0 mU/L; (iv) absence of medications known to interfere with thyroid hormone absorption or metabolism; (v) postoperative maintenance of biochemical euthyroidism (TSH ≥0.4 mU/L and ≤4.0 mU/L) during follow-up. Exclusion criteria included: (i) use of interfering pharmacological agents (e.g., estroprogestins, sucralfate, proton-pump inhibitors, calcium carbonate, carbamazepine, phenytoin, phenobarbital, iron supplements); (ii) gastrointestinal disorders associated with malabsorption (celiac disease, Crohn's disease, autoimmune gastritis); (iii) preoperative TSH <0.5 mU/L or >4.0 mU/L; (iv) evidence of persistent or recurrent malignant thyroid disease after surgery; (v) inability to complete follow-up or provide informed consent.

All patients included in the study received replacement-dose levothyroxine, in accordance with the approved clinical guidelines [75], without the intention of inducing suppressive therapy. Thyroid function tests and blood sampling were performed preoperatively and again at 3, 6, and 12 months post-surgery. As part of routine clinical practice, serum and plasma aliquots from each time point were stored at -80 °C in the institutional biobank. TSH levels were

measured using a solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000, DPC, Los Angeles, CA).

The study was conducted in accordance with the Declaration of Helsinki (1975, revised 2013). Ethical approval was obtained from the local ethics committee (protocol no. *Thyroidomics*). All participants provided written informed consent for the use of their clinical and biological data for research purposes.

Parameter	Overall (n=12)	Pre-surgery	Post-surgery	DIO2 Polymorphisms		QoL clusters	
				Variant (n=6)	*Wild type (n=6)*	*Cluster 1 (n=8)*	*Cluster 2 (n=4)*
Age (years)	69.2 ± 6.8	-	-	-	-	-	-
Sex (M/F	5/7	-	-	-	-	-	-
TSH (mU/L)	-	0.94 ± 0.70	1.22 ± 0.56	1.34 ± 0.53	0.96 ± 0.50	1.02 ± 0.52	1.40 ± 0.62
FT3 (pg/mL)	-	3.22 ± 0.39	3.11 ± 0.26	3.20 ± 0.38	3.07 ± 0.08	3.03 ± 0.13	3.35 ± 0.37
FT4 (pg/mL)	-	9.16 ± 1.73	12.65 ± 2.34	12.50 ± 1.42	13.53 ± 2.97	12.80 ± 2.23	13.20 ± 2.66
SF-36 domains (post-surgery)							
Physical functioning	-	-	-	-	-	76 ± 12	40 ± 34
Energy/fatigue	-	-	-	-	-	61 ± 20	33 ± 16
Emotional well-being	-	-	-	-	-	64 ± 22	40 ± 20
Social functioning	-	-	-	-	-	71 ± 19	46 ± 24
Pain	-	-	-	-	-	78 ± 22	52 ± 39
General health	-	-	-	-	-	63 ± 22	33 ± 16
Health change	-	-	-	-	-	63 ± 24	25 ± 25

Table.1 ***Clinical, biochemical, and questionnaire-derived data from the 12 patients included in the study****. Columns show overall means and values stratified by pre- and post-thyroidectomy phases, DIO2 p.Thr92Ala genotype (variant vs wild-type, calculated post-surgery), and postoperative quality-of-life (QoL) clusters identified through Gaussian Mixture Model (GMM) analysis. Data are expressed as*

mean ± standard deviation (SD). Thyroid function parameters (TSH, FT3, FT4) were measured before and after surgery under LT4 replacement therapy. SF-36 questionnaires were administered both pre- and post-surgery; however, only post-operative scores are reported here, as they showed significant clustering patterns. Higher SF-36 scores indicate better perceived well-being.

3.2 Health-Related Quality of Life Assessment (SF-36)

Health-related quality of life was evaluated using the 36-Item Short Form Health Survey (SF-36), a widely validated patient-reported outcome instrument originally developed within the Medical Outcomes Study [69]. The questionnaire consists of 11 questions comprising 36 items, generating scores across nine components, each scaled from 0 to 100, where higher scores correspond to better perceived quality of life. The SF-36 assesses the following domains: (i) Physical Functioning; (ii) Role Limitations due to Physical Health; (iii) Role Limitations due to Emotional Problems; (iv) Energy/Fatigue; (v) Emotional Well-Being; (vi) Social Functioning; (vii) Pain; (viii) General Health; (ix) Health Change. Item responses were coded, summed, and transformed following standard RAND-based scoring conventions [70], with assistance from the OrthoToolKit scoring platform (© OrthoToolKit, 2025). Scores range from 0 (worst possible health state) to 100 (best possible health state). The questionnaire was administered preoperatively and at 3, 6, and 12 months after thyroidectomy to monitor longitudinal changes in patient-reported outcomes.

For downstream analyses, seven domains were retained and the two role-limitation domains (*Role-Physical* and *Role-Emotional*) were excluded due to overlapping conceptual content and statistical redundancy with other scales.

3.3 Nucleic Acid Extraction and Purification

For each patient, serum and plasma samples were collected at baseline and at follow-up time points according to the study protocol. Peripheral blood samples for DNA extraction were also collected in EDTA tubes. All samples were stored at -80 °C until analysis.

Genomic DNA was extracted from EDTA blood samples using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Total RNA was extracted from the plasma samples using the SV Total RNA Isolation System (Promega, Madison, WI, USA), a rapid and efficient method for isolating purified, intact RNA. This system includes an on-column DNase treatment step to minimize genomic DNA contamination. The quantity and quality of the extracted DNA and RNA were assessed using two complementary approaches: spectrophotometric analysis with a NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and fluorometric quantification with Qubit™ DNA High Sensitivity (HS) and Qubit™ RNA HS Assay Kits on a Qubit™ Fluorometer (Thermo Fisher Scientific).

3.4 Single Nucleotide Polymorphism (SNP) Analysis of DIO2 Thr92Ala

Genotyping of the *DIO2 Thr92Ala* (rs225014, C/T transition) polymorphism was performed using a TaqMan™ SNP Genotyping Assay (Thermo Fisher Scientific, Assay ID: C__15819951_10) that targeted the context sequence TTGCCACTGTTGTCACCTCCTTCTG[C/T]ACTGGAGACATGCACCACACTGGAA.
PCR reactions were carried out on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific) with automated allelic discrimination analysis, performed using the QuantStudio™ Design and Analysis Software v1.5.1. Due to the low frequency of the Ala92Ala homozygous genotype in the cohort, and consistent with previous literature indicating that both heterozygous and homozygous Ala carriers exhibit similar phenotypic and functional consequences of impaired DIO2 activity, including higher BMI and fasting glucose levels compared with non-

carriers [76], patients were classified into two groups for downstream analyses: (i) wild-type (*Thr92Thr*) and (ii) variant carriers (*Thr92Ala* + *Ala92Ala*).

3.5 Gene expression analysis

3.5.1 Discovery phase: NanoString nCounter® transcriptomic profiling

Gene expression profiling was performed using the NanoString nCounter® platform (NanoString Technologies, Inc., Seattle, WA) and the *Human Metabolic Pathways Panel* (768-gene panel, catalog #XT-CSO-HMP1-12), according to the manufacturer's protocol. For each sample, 100–150 ng of total RNA was used as input. Fluorescently barcoded reporter probes and biotin-labeled capture probes were hybridized to the target mRNAs overnight in a thermal cycler. After hybridization, the samples were automatically processed on the nCounter Prep Station (NanoString Technologies, Inc., Seattle, WA) and loaded into NanoString sample cartridges according to the manufacturer's protocol. The subsequent acquisition of digital counts of probe-target complexes was conducted using the nCounter Digital Analyzer (NanoString Technologies, Inc., Seattle, WA).

3.6 Bioinformatic Analysis

All analyses were conducted in R (v4.3.1) using a comprehensive bioinformatics pipeline designed to investigate transcriptomic alterations and their biological significance in the context of thyroidectomy. Differential expression analyses were performed to identify genes modulated (i) by surgical intervention (pre- vs post-treatment), (ii) by the presence of specific genetic variants (DIO2 polymorphism), and (iii) across post-treatment patient subgroups derived from unsupervised clustering of quality-of-life (SF-36) domains. Enrichment analyses were performed to provide a functional interpretation of the deregulated gene sets. These analyses leveraged curated biological ontologies and protein interaction databases.

3.6.1 Differential Gene Expression Analysis

Differential gene expression analyses (DEG) were performed using the DESeq2 [77] package (v1.40.2). Prior to analysis, genes with insufficient read counts (less than 10 in fewer than 3 samples) were excluded to mitigate the impact of low-abundance transcripts. After DEG analysis, p-values were adjusted for multiple testing using the Benjamini–Hochberg procedure to control the false discovery rate (FDR). Genes with FDR < 0.05 and an absolute $\log_2$ fold change greater than 1 were considered significantly differentially expressed.

Three main comparisons were carried out. First, a paired intra-patient analysis was implemented to identify transcriptomic changes associated with thyroidectomy by comparing post-treatment to pre-treatment samples from the same individuals. This analysis accounted for inter-individual variability by incorporating patient ID as a blocking factor in the model design. Second, to assess the transcriptional impact of genetic variability, patients were stratified according to the presence of a DIO2 polymorphism, and differential expression analyses were conducted separately for pre-treatment and post-treatment groups. Finally, gene expression profiles were compared between post-treatment subgroups identified through unsupervised clustering of quality-of-life (SF-36) domains. In all comparisons, differential expression results were visualized using volcano plots generated with the EnhancedVolcano package 1.26.0, allowing for intuitive representation of both statistical significance and magnitude of change.

3.6.2 Gene Set Enrichment Analysis (GSEA)

Gene set enrichment analysis was conducted to contextualize the biological relevance of the deregulated genes identified in the differential expression analyses. Enrichment was performed using g:Profiler (version 114, April 2025, April 2025), which queries multiple functional databases integrated within the platform.. These included Gene Ontology-Biological Process (annotations current as of May 2024), KEGG (v114, April 2025), and Reactome (v88, April

2025). These resources were used to identify significantly enriched biological pathways and molecular processes associated with the observed transcriptional changes. The gene sets used for enrichment, included the significantly differentially expressed genes from the paired intra-patient comparison, the genotype-based analyses, and the clustering-derived subgroups.

To enhance interpretability, enrichment results from g:Profiler were jointly evaluated, providing both functional and interaction-level perspectives. Visual representations included bar plots, where bar length reflected the number of genes annotated to each term and color intensity encoded statistical significance ($-\log_{10}$ *p*-value). In addition, circos plots were generated to display gene–term associations and highlight shared functional pathways across the enriched networks, integrating annotation from GO, KEGG, Reactome and TF databases.

3.7 Statistical Analysis

Unsupervised clustering was applied to scaled clinical questionnaire scores (post-treatment only) using Uniform Manifold Approximation and Projection (UMAP) for dimensionality reduction and Gaussian Mixture Models (GMM) for cluster identification. Dimensionality reduction was performed with n_neighbors = 3 and min_dist = 0.3 using the umap package v0.2.10, and clustering was executed via the mclust v6.1.1 library.

Inter-cluster differences across clinical metrics were assessed using Wilcoxon rank-sum tests and boxplots were generated to visualize group distributions using GraphPad Prism. Cluster labels obtained from this unsupervised analysis were subsequently used to stratify transcriptomic data for DEG analysis as described above. All statistical tests were two-sided, and a significance threshold of $p < 0.05$ was applied unless stated otherwise.

4. Results

4.1 Paired differential expression before and after thyroidectomy

A total of 12 patients met the inclusion criteria (Table 1) and underwent transcriptomic profiling using the NanoString platform to evaluate gene expression changes associated with thyroidectomy and levothyroxine replacement therapy. An intra-patient paired analysis was conducted to capture the molecular response to surgery and subsequent LT4 treatment, using each patient as their own control. This approach enabled direct comparison of gene expression profiles before and after thyroidectomy, aiming to identify shared transcriptional alterations potentially linked to subclinical tissue hypothyroidism despite normalization of circulating thyroid hormones (TSH, FT3, and FT4).

Differential expression analysis applied to postoperative samples, independently of *DIO2* genotype or clinical cluster membership, identified a set of genes significantly upregulated (**Figure 1**). Among the six genes identified were *THBS1* (Thrombospondin-1), *ITGB1* (Integrin β1), *CCL5* (C–C motif chemokine ligand 5), *PTGS1* (prostaglandin-endoperoxide synthase 1), *GRAP2* (Grb2-related adapter protein 2), and *CTSA* (cathepsin A).

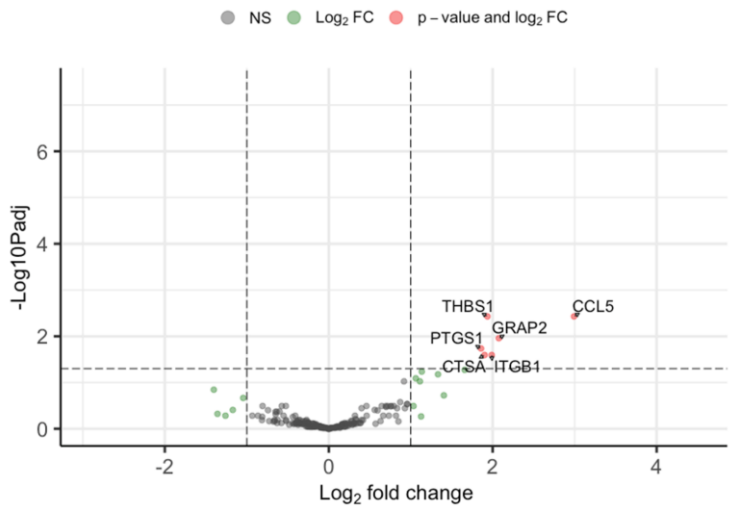

Figure 1 Volcano Plot of Paired Differential Gene Expression (Post- vs. Pre-Thyroidectomy). *Results of paired differential gene expression analysis comparing samples collected before and after total thyroidectomy. Thresholds of log_2 fold change ≥1 and p-value <0.05 are displayed as vertical and horizontal grey dotted lines respectively. Genes highlighted in red meet both significance criteria including THBS1, ITGB1, CCL5, GRAP2, PTGS1, and CTSA.*

4.1.1 GSEA-based functional analysis before and after thyroidectomy

To further explore the functional implications of differentially expressed genes (DEGs), a Gene Set Enrichment Analysis (GSEA) was performed using the g:Profiler platform. This analysis revealed significant enrichment of interconnected biological processes related to *chronic inflammatory response*, *metabolic adaptation*, and *ECM remodeling* (**Figure 2A**). Notably, "*cellular response to fibroblast growth factor stimulus*" (GO:0044344) involved *THBS1, ITGB1*, and *CCL5* (**Figure 2B**). The term "*Cell-cell adhesion mediated by integrin*" (GO:0033631), enriched by *ITGB1* and *CCL5*, reflects changes in adhesion-associated pathways compatible with a stressed systemic micro-environment. The GO term of "*Positive regulation of PI3K/AKT signal transduction*" (GO:0051897), annotated for THBS1, ITGB1, and *CCL5*, also emerged among enriched processes.

Reactome pathway analysis highlighted "*Signaling by Receptor Tyrosine Kinases*" (R-HSA-9006934), with *GRAP2* contributing according to its annotated protein–interaction network. The term "*Syndecan interactions*" (R-HSA-3000170), enriched by THBS1 and ITGB1, corresponded to ECM-related interaction pathways. "*Metabolism of Lipids*" (R-HSA-556833), associated with *PTGS1* and *CTSA*, was also significantly enriched. Finally, transcription factor motif enrichment (TRANSFAC) identified putative STAT5B binding sites in the promoter regions of *CCL5* and *CTSA*. This represents an exploratory computational result and indicates possible up-stream regulation requiring further confirmation.

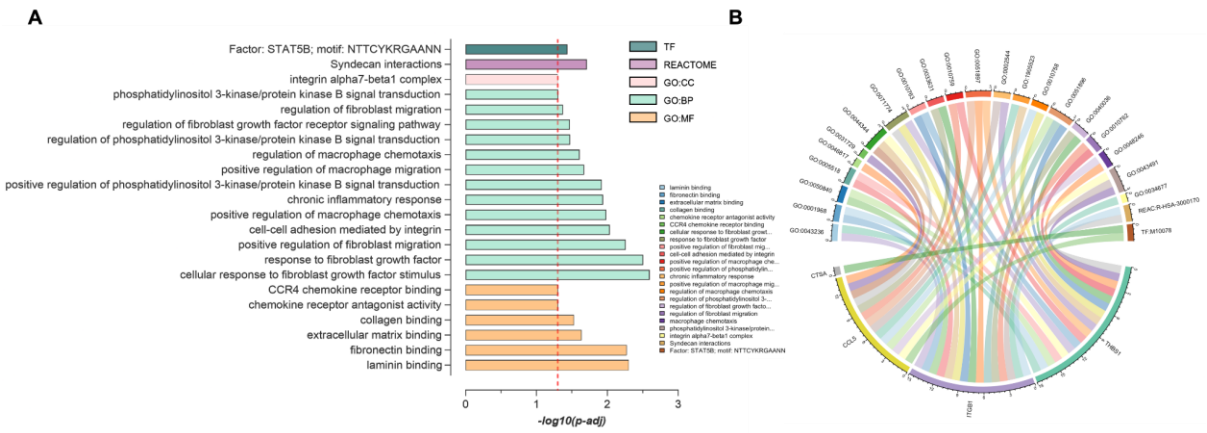

Figure 2. Gene Set Enrichment Analysis (Post- vs. Pre-Thyroidectomy). *(**A**) Bar plot of enriched Gene Ontology (GO), Reactome (REAC), and transcription factor (TF) terms, showing the most significant functional categories associated with differentially expressed genes (DEGs). Bars represent -log10(adjusted p-value), with color codes indicating the database source. (**B**) Circos plot depicting the connections between DEGs (outer circle, bottom half) and enriched functional categories (outer circle, top half). Links highlight gene–term associations, illustrating the contribution of individual genes (e.g., THBS1, ITGB1, CCL5, CTSA) to multiple enriched pathways, particularly those related to extracellular matrix interactions, chemokine signaling, and fibroblast responses.*

4.2 Genotype-associated transcriptional differences in *DIO2* variant carriers

Genotyping showed that six patients carried the wild-type *DIO2* allele (*Thr92Thr*), while the remaining six patients were variant carriers, carrying at least one Ala allele (*Thr92Ala*/ *Ala92Ala*). This stratification enabled us to investigate whether the *p.Thr92Ala* polymorphism of the DIO2 gene, modulated the transcriptional response to thyroidectomy and subsequent LT4 replacement therapy. At baseline, DEG analysis, comparing variant carriers and wild-type patients, revealed no significant differences (padj > 0.05), indicating a comparable pre-surgical transcriptional profile between the two groups.

After thyroidectomy and hormonal stabilization, a distinct transcriptional divergence emerged (**Figure 3**). Variant carriers showed significant downregulation of several genes, including those related to mitochondrial detoxification (*ALDH2),* oxidative and inflammatory stress responses (*MAPK8*, *LY96*), and extracellular matrix integrity (*LAMB1*). Concurrently, upregulation was detected in *NDUFB4* (mitochondrial respiratory complex I subunit) and *RPS6KB1* (mTOR pathway effector). Together, these results indicate the presence of genotype-associated transcriptional differences after thyroidectomy and LT4 stabilization, highlighting molecular heterogeneity that is not clearly evident from standard biochemical markers.

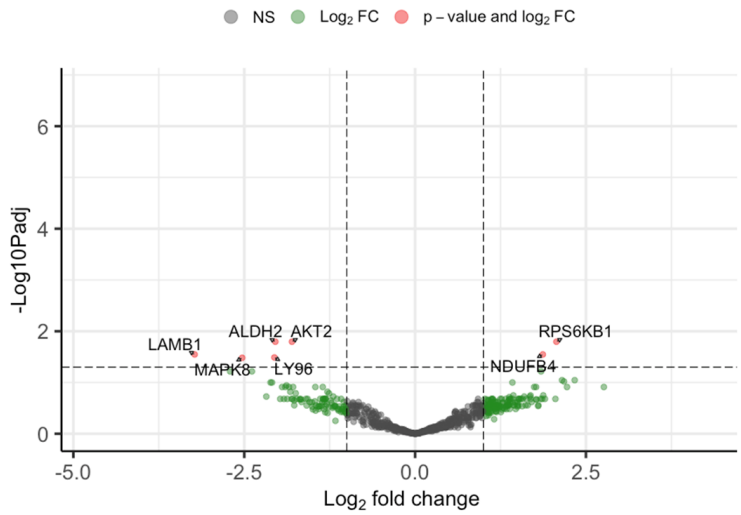

Figure 3. Differential Gene Expression in DIO2 (rs225014) polymorphism carriers compared to Wild-Type patients following LT4 therapy. *The volcano plot displays genes that are differentially expressed in DIO2 polymorphism carriers relative to wild-type patients, based on postoperative samples collected after hormonal stabilization under LT4 treatment. Thresholds of log_2 fold change ≥1 and p-value <0.05 are displayed as vertical and horizontal grey dotted lines respectively. Genes meeting both statistical and effect-size criteria are highlighted in red.*

4.2.1 GSEA-based functional analysis by DIO2 polymorphism

To functionally characterize the DEGs identified in DIO2 variant carriers, a gene set enrichment analysis was performed using g:Profiler, querying GO Biological Process, KEGG, and Reactome annotations (**Figure 4**). GO enrichment highlighted a significant over-representation of "*Protein serine/threonine kinase activity"* (GO:0004674) (**Figure 4A**), which included *AKT2* and *MAPK8* (both downregulated in variant carriers) and *RPS6KB1* (upregulated) (**Figure 4B**). The GO term "*Long-chain fatty acid import into cell*" (GO:0044539) was also enriched, involving *AKT2* and *RPS6KB1*. Within KEGG, significant enrichment was detected for "*Endocrine resistance*" and "*Insulin resistance*", primarily driven by the downregulation of *AKT2* and *MAPK8*. Additional KEGG pathways included "Focal adhesion" and "Lipid and atherosclerosis", with *AKT2, MAPK8,* and *LAMB1* significantly

downregulated. Reactome enrichment further identified "*Syndecan interactions*," "*Apoptosis*" (R-HSA-109581), and "*Activation of BH3-only proteins*" (R-HSA-114452), reflecting differential expression of genes involved in adhesion dynamics and apoptotic signaling. Upregulation of *NDUFB4*, a structural component of mitochondrial respiratory complex I, contributed to enrichment of mitochondrial and lipid-related pathways, including "*Lipid and atherosclerosis*" and "*Non-alcoholic fatty liver disease (NAFLD)*". Collectively, enriched GO, KEGG, and Reactome terms indicate that DEGs associated with the *DIO2* polymorphism map to pathways involved in kinase activity, endocrine- and insulin-resistance, lipid import, focal adhesion, mitochondrial processes, and apoptosis-related signaling.

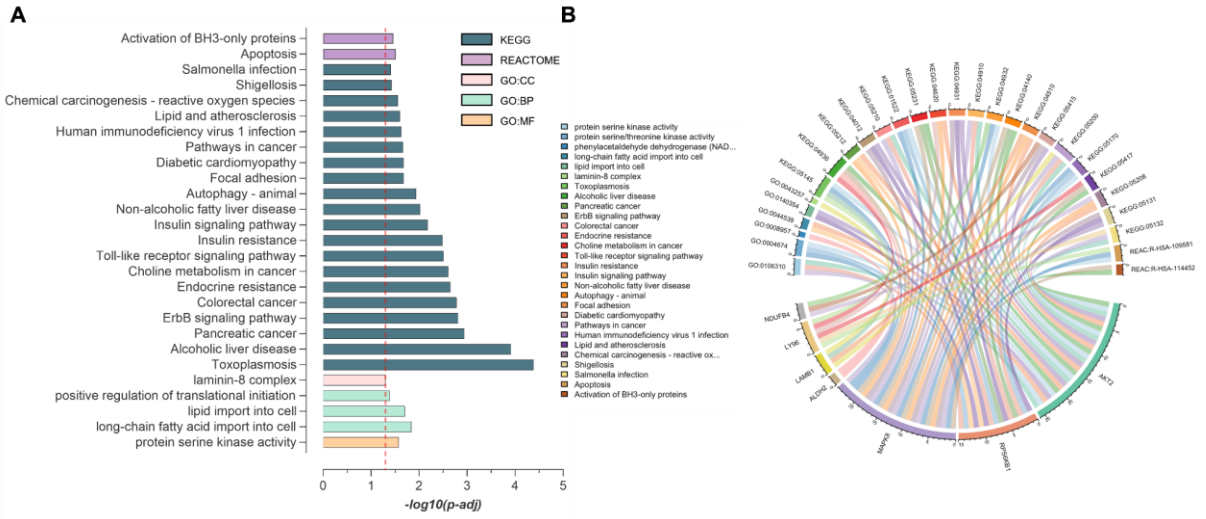

Figure 4. Gene Set Enrichment Analysis in patients stratified by DIO2 genotype. *(**A**) Bar plot of enriched Gene Ontology (GO), KEGG, and Reactome (REAC) terms, showing the most significant functional categories associated with differentially expressed genes (DEGs) in DIO2-polymorphic patients. Bars represent –log10(adjusted p-value), with color codes indicating the database source. (**B**) Circos plot depicting the connections between DEGs (outer circle, bottom half) and enriched functional categories (outer circle, top half). Links highlight gene-term associations, illustrating the contribution of individual genes to multiple enriched pathways.*

4.3 Transcriptomic clustering and symptom profiles

Whether surgery and subsequent LT4 therapy could affect patients' quality of life, a parameter that currently represents the only clinical tool for correlating subjective symptoms with possible peripheral hypothyroidism, was investigated. Unsupervised analysis of post-operative SF-36 questionnaires revealed the presence of two clinically distinguishable subgroups, identified through UMAP dimensionality reduction followed by Gaussian Mixture Model (GMM) clustering: Cluster 1 (n=8), characterized by preserved quality of life, and Cluster 2 (n=4), showing significantly lower scores in domains such as vitality/fatigue, social functioning, pain, and general health (**Figure 5**).

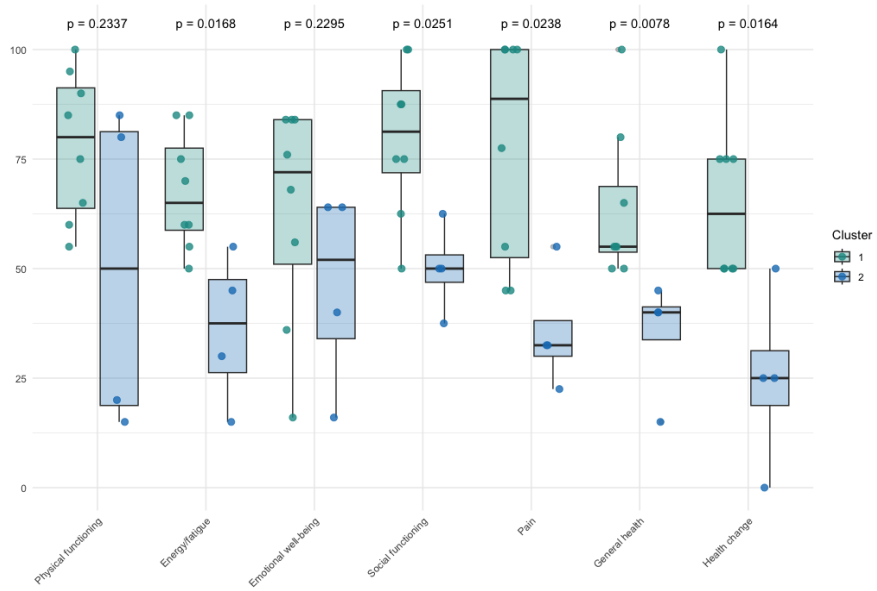

Figure 5. Distribution of SF-36 quality-of-life domain scores in post-thyroidectomy patients, stratified by unsupervised clustering analysis*. Boxplots display the median, first and third quartiles, and individual outliers for each domain, with patients assigned to Cluster 1 (n=8; green) or Cluster 2 (n=4; blue). Scores range from 0 (worst perceived quality of life) to 100 (best). Statistical analysis was performed using the Wilcoxon rank-sum test, and p-values are reported above each domain.*

The possibility that clinical or genetic variables, such as sex or the *DIO2* polymorphism, could account for this differentiation was investigated, but no statistically significant association was found ($p>0.05$). This suggested the presence of an alternative underlying mechanism for the divergent post-surgical clinical perception. DEG analysis between the two clusters revealed a specific transcriptional profile in Cluster 2 patients. This profile was characterised by the upregulation of genes associated with oxidative stress (*MSRB2*), cerebral metabolism (*GLUL*), neuroinflammation (*CTSS*) and energy dysregulation (*CAB39*) (**Figure 6**).

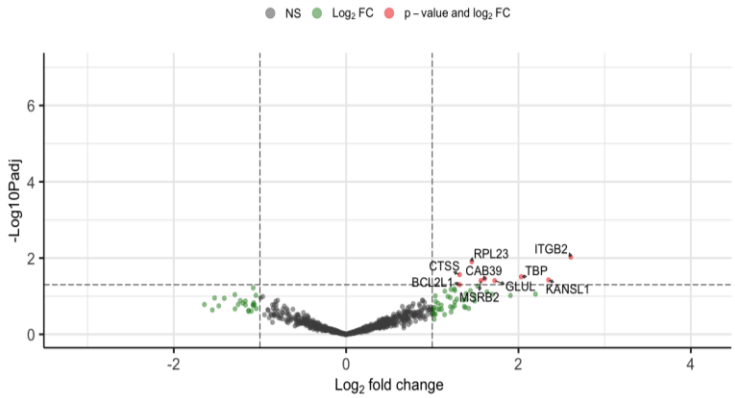

Figure 6. Volcano plot of differential gene expression between QoL-derived patient clusters. *The volcano plot represents differential expression analysis between the two patient subgroups defined by GMM clustering of SF-36 scores (Cluster 2 vs. Cluster 1) in post-surgical samples. Analysis was performed using the DESeq2 package with the model "~ gmm." The x-axis shows log_2 fold change (Cluster 2 vs. Cluster 1), while the y-axis displays $–log_{10}$ adjusted p-values (padj). Red points indicate genes with $log_2FC \geq |1|$ and nominal $p < 0.05$ that did not retain statistical significance after multiple-testing correction; green points mark genes meeting the fold-change but not the p-value criterion; gray points (NS) represent non-significant genes.*

Although this molecular signature did not reach statistical significance following correction for multiple testing, it was consistent with the reported clinical symptoms, suggesting the presence

of a shared physio-pathological core link to hypothyroidism symptoms following thyroidectomy. This condition cannot be detected by serum markers alone, but can be identified through transcriptomic biomarkers. This provides the basis for a personalised approach to the postoperative management of patients who have undergone thyroidectomy.

4.3.1 GSEA-based functional profiling of SF-36-derived patient clusters

Transcriptomic analysis revealed a distinctive molecular profile in Cluster 2, characterized by the upregulation of the following nine genes: *GLUL, BCL2L1, TBP, CTSS, ITGB2, CAB39, RPL23, MSRB2* and *KANSL1*. GO enrichment analysis identified two significantly over-represented pathways: '*Enzyme bindin*g' (GO:0019899) and '*Response to thyroid hormone*' (GO:0097066). Analysis of transcription factor binding sites using the TRANSFAC database revealed significant enrichment for PU.1, with predicted binding sites in seven of the upregulated genes (*GLUL, TBP, CTSS, ITGB2, CAB39, RPL23* and *MSRB2*). This suggests a coordinated transcriptional program in Cluster 2 patients.

5. Discussion

Patients undergoing total thyroidectomy and treated with LT4 replacement therapy, are usually considered biochemically euthyroid; however, a substantial subset of them continue to experience symptoms consistent with hypothyroidism despite normal TSH levels. Our previous findings demonstrated that thyroidectomized patients carrying the DIO2 Thr92Ala polymorphism are at higher risk of reduced FT3 concentrations during LT4 monotherapy, supporting the hypothesis of a functional, tissue-level hypothyroidism in this subgroup [46,72]. To explore this condition, we previously performed a serum proteomic analysis comparing thyroidectomized patients with “reduced” versus “stable” FT3 levels and healthy controls. That study revealed a differential abundance of proteins involved in complement activation, coagulation cascades, and lipoprotein remodeling, pointing to sustained inflammatory and

vascular activation in LT4-treated patients with lower FT3 availability [74]. These proteomic findings indicated that immune and metabolic dysregulation may represent a systemic correlate of inadequate intracellular TH signaling. Building on these results, the present study extends the investigation from the proteome to the circulating transcriptome to identify *up-stream molecular mechanisms* and *transcriptional networks* potentially responsible for these systemic alterations. The analysis of plasma-derived mRNA profiles, performed with Nanostring platform, revealed distinct transcriptional programs associated with extracellular matrix (ECM) remodeling, neuro-immune activation, mitochondrial metabolic stress, and impaired stress response regulation. We hypothesized that these circulating signals, likely mirror gene expression perturbations in peripheral tissues that are highly responsive to TH availability, including skeletal muscle, adipose tissue, and the nervous system.

Although all patients achieved biochemical euthyroidism on LT4 therapy , the persistence of symptoms compatible with hypothyroidism suggests that circulating mRNA signatures reflect peripheral metabolic and inflammatory imbalances that persist despite normalized serum hormone concentrations. This supports the concept of a circulating transcriptional fingerprint of insufficient intracellular TH action. The transcriptional landscape uncovered by this analysis, converges onto four major biological domains that describe the long-term systemic adaptations, occurring after thyroidectomy: (i) ECM-integrin and PI3K/AKT/mTOR signaling; (ii) immune and neuro-immune activation; (iii) mitochondrial and neuro-metabolic stress; and (iv) survival of stressed but dysfunctional cells.

(i). ECM remodeling and integrin-driven signaling reorganization

The first transcriptional domain emerging from the circulating mRNA profiles involves ECM remodeling and integrin-dependent signaling, two processes that are central to cellular adhesion, communication, and tissue adaptation after injury. The coordinated upregulation of

THBS1, *ITGB1*, *ITGB2*, and the ECM-associated protease *CTSA* suggests a persistent reorganization of the extracellular environment, after thyroidectomy.

THBS1 (thrombospondin-1) and ITGB1 (integrin β1) are key regulators of ECM remodeling and mechano-transduction [78]. THBS1 binds multiple β-integrins and modulates endothelial adhesion, migration, and angiogenic balance, acting as an anti-angiogenic mediator in stressed microenvironments [79,80]. Integrin β1, activates PI3K-dependent signaling and cytoskeletal pathways involved in cell survival and matrix sensing [81,82], a mechanism also supported by recent reports linking ITGB1 activity to PI3K-AKT remodeling under metabolic or inflammatory stress [83–85]. The concomitant upregulation of THBS1 and ITGB1 thus likely reflects activation of an ECM–integrin axis indicative of sustained remodeling pressures. This pattern is compatible with a microenvironment characterized by incompletely resolved reparative responses and altered angiogenic signals, an effect previously described in states of reduced thyroid hormone action [86]. This interpretation aligns with our earlier proteomic findings reporting activation of complement and coagulation cascades in LT4-treated thyroidectomized patients [74], pathways that are mechanistically interconnected with ECM turnover and vascular integrity [87].

Of particular interest is the upregulation of LAMB1 (laminin β1), a core structural component of basement membranes and a key regulator of neuro-glia interactions. Laminin β1 participates in the assembly of laminin heterotrimers that shape the ECM and influence cellular adhesion, signaling, and synaptic organization within the CNS [88,89]. Recent work has shown that LAMB1 is highly expressed in cortical regions and is dynamically regulated under neural stress or injury, with reduced expression contributing to maladaptive plasticity, enhanced pain sensitivity, and anxiety and depression-like phenotypes through integrin β1-dependent cytoskeletal remodeling [90–92].

In parallel, thyroid hormones are known to modulate astrocyte morphology, maturation [93–95], and extracellular matrix organization [96]. Experimental evidence indicates that TH availability regulates astrocytic differentiation, radial glia-to-astrocyte transition, and ECM protein expression, thereby influencing neuron-glia crosstalk [97,98]. Taken together, the observed downregulation of circulating LAMB1 transcripts may thus reflect impaired ECM maintenance and disrupted glia-ECM-integrin signaling in a TH-sensitive context. Such reduction could mirror reduced neurovascular structural support and altered glial homeostasis following thyroidectomy, despite biochemical euthyroidism. Concurrently, *AKT2* and *MAPK8* were downregulated, while *RPS6KB1* and *CAB39* were upregulated. This pattern suggests a shift away from canonical AKT/MAPK trophic signaling [99] toward compensatory activation of mTOR- and AMPK-related pathways, typically engaged under metabolic stress and limited intracellular T3 signaling [100–102]. This reorganization may represent an adaptive attempt to stabilize cellular energy balance under conditions of impaired TH signaling.

Taken together, these findings outline a persistent ECM-integrin-PI3K-mTOR axis, indicating a chronic state of reparative and metabolic adaptation rather than complete homeostatic recovery. The fact that these transcriptional alterations are detectable in circulating mRNA, months after surgery and under biochemical euthyroidism, supports the concept of functional hypothyroidism, in which tissues remain metabolically inflexible and less responsive to TH-mediated signals.

(ii). Immune and neuro-immune activation: a sustained inflammatory loop

A second transcriptional domain emerging from the circulating mRNA profile involves persistent low-grade immune activation. The concomitant upregulation of *CCL5, CTSS, ITGB2,* and the transcriptional regulators *RELA* (NF-κB) and *BCL2L1* points to the establishment of a chronic inflammatory network interconnected with the ECM-integrin axis.

CCL5, represents a central node in this module. In the CNS, CCL5 is constitutively produced by microglia and astrocytes and signals predominantly through *CCR5*, modulating leukocyte recruitment [103], glial activation, and neuroimmune communication [104,105]. Experimental models have shown that activated microglia release CCL5 as part of a feed-forward inflammatory loop that contributes to neuronal dysfunction, excitatory imbalance, and, under chronic conditions, neurodegenerative-like changes [106]. Elevated CCL5-CCR5 signaling has also been implicated in cognitive impairment, neuroinflammation, and postoperative neurocognitive dysfunction (POCD), where microglial activation drives excessive *CCL5* release and CCR5-mediated neuronal injury [107]. Importantly, thyroid hormone insufficiency, even when limited to intracellular or tissue-level defects, has been shown to promote microglial M1 polarization, reduce BDNF expression, and suppress PI3K-AKT signaling, thereby amplifying pro-inflammatory microglial states [108]. This is highly relevant to the present findings, as *THBS1*, one of the most upregulated genes in our cohort, has been specifically reported to be increased in M1-polarized macrophages and microglia [109], reinforcing the association between reduced TH action and pro-inflammatory myeloid activation.

CTSS, a lysosomal cysteine protease uniquely able to retain enzymatic activity at neutral pH [110], operates in both intracellular and extracellular compartments, thereby linking antigen processing to matrix remodeling. Intracellularly, *CTSS* participates in MHC class II maturation and antigen presentation in antigen-presenting cells [111,112]. Extracellularly, due to lysosomal exocytosis, *CTSS* can remodel the extracellular matrix, activate protease-activated receptors, and promote cytokine release [113]. *CTSS* is particularly relevant in chronic inflammatory states: cysteine cathepsins, including *CTSS*, process ECM proteins as well as cytokines, chemokines, and adhesion molecules, contributing to sustained tissue remodeling [114]. Elevated extracellular cathepsin activity characterizes multiple inflammatory and cardiovascular disorders and often reflects cytokine-driven transcriptional upregulation or imbalance with endogenous inhibitors

[115–117]. IL-1α and TNF-α, for example, enhance CTSS expression and secretion in human chondrocytes [118]. Recent studies indicate that *CTSS* acts as an amplifier of inflammatory pathways by regulating NF-κB/MAPK transcriptional activity, exposing active domains of downstream signaling proteins, and releasing inflammatory mediators [119]. Stress stimuli-psychological, metabolic, or infectious-converge on *CTSS* overexpression, producing a persistent pro-inflammatory microenvironment. In parallel, CTSS transcription is stimulated by stress-responsive transcription factors such as *PU.1* [120], consistent with our evidence of PU.1 motif enrichment among upregulated genes, including CTSS.

Interestingly, the observed downregulation of *LY96* (*MD-2*) may indicate a compensatory reduction in sensitivity to endotoxin-like ligands. MD-2 is an essential co-receptor for Toll-like receptor 4 (TLR4), and the TLR4/MD-2 complex is required for physiological recognition of LPS and initiation of downstream innate immune signaling [121]. Thus, reduced *LY96* expression in circulating transcripts may reflect an adaptive attempt to dampen TLR4 engagement in the context of chronic low-grade inflammation. Despite this potential desensitization at the receptor level, pro-inflammatory transcriptional activity appears to remain active, as indicated by the upregulation of *RELA* (NF-κB). *RELA/p65* is a central effector of NF-κB-mediated cytokine programs and is widely recognized as a key driver of sustained inflammatory responses when resolution mechanisms fail [122]. The simultaneous induction of *BCL2L1* (*BCL-xL*) further reinforces this scenario: *BCL-xL* is an anti-apoptotic mitochondrial protein that inhibits BAX/BAK-mediated mitochondrial outer membrane permeabilization (MOMP), thereby preventing apoptosis and promoting persistence of metabolically active but stressed cells [123,124]. Elevated *BCL-xL* expression has been mechanistically linked to long-term survival of senescent or chronically activated cells, delaying their clearance and contributing to sustained inflammatory microenvironments [124].

Taken together, these findings suggest the presence of a self-sustaining inflammatory state. This chronic immune priming, keeps immune cells activated and aligns with neuro-immune evidence linking prolonged cytokine signaling to fatigue, pain hypersensitivity, and mood disturbances [125]. Such mechanisms offer a plausible explanation for the persistent symptoms reported by thyroidectomized patients.

(iii). Mitochondrial and neuro-metabolic reprogramming under oxidative stress

In parallel with immune activation, a third transcriptional module encompasses alterations in mitochondrial dynamics, redox balance, and glial metabolism. The coordinated upregulation of *PTGS1, NDUFB4, RPS6KB1, MSRB2,* and *GLUL,* together with the downregulation of *ALDH2*, delineates a systemic adaptation to oxidative and metabolic stress following thyroidectomy.

Mitochondria represent the major endogenous source of reactive oxygen species (ROS), particularly at Complex I, Complex III, and other redox-active sites of the electron transport chain (ETC) [126]. Excess electron leakage at these sites produces superoxide ($O_2^{\bullet -}$) and downstream oxidants such as H_2O_2 and •OH, initiating oxidative damage to lipids, proteins, and mitochondrial DNA. The observed upregulation of NDUFB4, a structural component of mitochondrial Complex I, may therefore reflect increased ETC flux, a compensatory attempt to sustain ATP production, that can also amplify mitochondrial ROS generation under stress conditions [127].

In parallel, PTGS1 (COX-1), traditionally known for prostaglandin synthesis, can act as an additional enzymatic source of ROS, particularly within vascular and inflammatory contexts [128]. ROS derived from COX enzymes can trigger systemic inflammatory responses and propagate oxidative stress via feed-forward loops involving NADPH oxidase and mitochondrial ROS production [129].

At the detoxification level, the downregulation of ALDH2 is notable. ALDH2 is a mitochondrial aldehyde dehydrogenase essential for clearing reactive aldehydes formed during lipid peroxidation. Reduced ALDH2 activity impairs aldehyde detoxification, thereby exacerbating oxidative injury, promoting neurodegenerative processes, and amplifying mitochondrial dysfunction [130,131]. Experimental evidence shows that ALDH2 deficiency leads to oxidative lipid adduct accumulation, mitochondrial damage, neuronal loss, and enhanced vulnerability to stress-related metabolic dysfunctions [130].

Beyond the mitochondrial ROS burden, several stress-response genes showed coordinated modulation, indicating activation of protective metabolic programs. MSRB2, a mitochondrial methionine-sulfoxide reductase, emerged as a key component of this response. MSRB2 is localized to the mitochondrial matrix and plays a central role in reversing oxidative methionine modifications on damaged proteins [132,133]. Its upregulation in our cohort therefore likely reflects an attempt to maintain mitochondrial proteostasis and prevent the accumulation of oxidized proteins, an event known to accelerate cellular senescence and neurodegenerative vulnerability [134,135].

In parallel, *RPS6KB1* (S6K1), a downstream effector of mTORC1, was upregulated, suggesting the engagement of compensatory growth and metabolic pathways [136]. Upregulation of *RPS6KB1* in a context of impaired AKT/MAPK trophic signaling, is consistent with a shift toward alternative metabolic routes aimed at stabilizing energy production under conditions of suboptimal intracellular T3 signaling.

The induction of GLUL (glutamine synthetase) further connects this module to astrocyte-dependent neuro-metabolic regulation. GLUL, expressed almost exclusively in astrocytes, converts glutamate to glutamine, thereby preventing excitotoxicity and sustaining neuronal mitochondrial metabolism [137]. Through this reaction, astrocytes also detoxify ammonia, an

essential process given that the CNS lacks a functional urea cycle and relies on astrocytes to buffer neurotoxic ammonium through glutamine synthesis [138]. Increased *GLUL* expression in plasma mRNA may therefore reflect astrocytic metabolic activation, aimed at counteracting oxidative stress, maintaining neurotransmitter cycling, and protecting neurons from glutamate and ammonia overload.

Together, the coordinated upregulation of MSRB2, RPS6KB1, and GLUL, along with the reduction in *ALDH2*, outlines a systemic state of mitochondrial hyperactivation and redox-stress compensation. Altogether, this transcriptomic pattern suggests metabolic inflexibility and neuro-energetic strain, offering a mechanistic explanation for cognitive slowing and fatigue reported by many thyroidectomized patients.

(iv). Failure of cellular clearance and maladaptive survival signaling

A fourth transcriptional domain converges downstream of inflammatory and metabolic stress and is characterized by impaired apoptotic activation and selective survival of damaged cells. The simultaneous downregulation of *MAPK8* (JNK1) and the upregulation of *BCL2L1* and *MSRB2* outline an anti-apoptotic program that favors survival of dysfunctional cellular populations rather than their clearance.

MAPK8 (JNK1) is a stress-activated kinase that normally promotes apoptosis when its activation is strong or sustained, particularly through induction of mitochondrial outer membrane permeabilization (MOMP) and activation of BH3-only pro-apoptotic pathways [139]. In this context, the downregulation of MAPK8 observed in our patients suggests a reduced ability to initiate stress-induced apoptosis, favoring the survival of damaged or bioenergetically compromised cells. In parallel, *BCL2L1*, one of the core anti-apoptotic members of the BCL2 family, is upregulated. BCL-xL is essential for inhibiting MOMP by neutralizing BH3-only proteins and preventing BAX/BAK pore formation at the outer mitochondrial membrane [140,141].

Through this mechanism, BCL-xL blocks cytochrome c release and caspase activation, promoting survival even under conditions of oxidative and metabolic injury [142]. Persistent BCL2L1 expression is a hallmark of cells that resist apoptosis and accumulate in stressed tissues, including senescent and metabolically impaired cell populations.

MSRB2, a mitochondrial methionine-sulfoxide reductase, is included in this module because of its established role in protecting stressed cells from oxidative injury. Its upregulation therefore likely reflects a compensatory mechanism that preserves mitochondrial proteostasis. However, partial or chronic activation of this pathway can permit the survival of dysfunctional, oxidatively damaged cells, a phenomenon known to promote paracrine inflammation and tissue decline [143].

Together, these transcriptional changes support a state in which apoptotic thresholds are elevated and stress-damaged cells acquire prolonged survival capacity. Such cells, often functionally impaired yet metabolically active, can generate ROS, release inflammatory mediators, and disturb local signaling networks, features consistent with senescent-like cellular persistence. In the postoperative thyroidectomy setting, this maladaptive survival program may contribute to chronic low-grade inflammation, impaired tissue renewal, and prolonged recovery dynamics despite biochemical euthyroidism.

In summary, this module completes the physiopathological framework emerging from the circulating mRNA transcriptome: a systemic environment in which survival pathways predominate over regenerative turnover, leading to prolonged stress adaptation rather than full restoration of physiological equilibrium.

5. Conclusion

This study provides the first integrated characterization of the circulating transcriptome in humans after total thyroidectomy for euthyroid benign multinodular disease under levothyroxine monotherapy. By adopting a paired before and after surgery design and extending evaluation beyond conventional biochemical measurements, we uncovered molecular perturbations that are systematically overlooked when thyroid function is assessed exclusively through serum TSH and thyroid hormone levels. Despite biochemical euthyroidism, all patients exhibited coherent transcriptional alterations across several biological domains, indicating that serum normalization does not necessarily reflect restored endocrine homeostasis at the tissue level.

Four interconnected molecular domains emerged. These alterations were observed uniformly across the cohort but were further modulated by genetic background: carriers of the DIO2 Thr92Ala variant demonstrated a distinct transcriptional phenotype. In parallel, patient stratification through SF-36–derived clusters revealed that individuals with persistent postoperative symptoms display transcriptional signatures enriched for oxidative stress, astrocyte-metabolic pathways, and inflammatory mediators, providing a molecular correlate to their clinical phenotype.

Collectively, these findings demonstrate that circulating mRNA profiling can detect subtle but biologically meaningful deviations in thyroid hormone-responsive pathways that remain invisible to standard thyroid function tests. The concept of biochemical euthyroidism is therefore insufficient to capture the full spectrum of postoperative thyroid hormone action, particularly in patients who rely entirely on peripheral T4-to-T3 conversion.

From a *translational perspective*, this work lays the groundwork for developing molecular biomarkers aimed at identifying thyroidectomized patients at risk of persistent symptoms

despite adequate LT4 therapy. Future studies with larger cohorts and targeted validation of key transcripts will be essential to refine these findings and pave the way toward precision approaches that incorporate genetic background, intracellular TH signaling, and molecular signatures alongside traditional biochemical parameters. Ultimately, the transcriptional fingerprint described here underscores the need to move beyond TSH-centric paradigms and to develop therapeutic strategies that more effectively restore true tissue-level euthyroidism in LT4-treated patients.

Limitation

The principal limitation of this work is the relatively small, monocentric cohort, which constrained statistical power while nonetheless revealing coherent and biologically convergent transcriptomic signals. In addition, circulating mRNA captures systemic rather than tissue-specific transcriptional activity, although its strong consistency with known thyroid hormone-responsive pathways supports its interpretative relevance. Finally, pathway and transcription-factor inferences derived from enrichment analyses remain mechanistic hypotheses and will require validation through targeted experimental studies.

Funding sources

Funded by the European Union-Next Generation EU, PNRR MUR M4C2 Inv. 1.5 of the National Recovery and Resilience Plan (PNRR), project ECS00000017 Tuscany-Health Ecosystem Spoke6-CUP B63C22000680007

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