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EFFECTS OF THYROTROPIN AND THYROID HORMONES ON THE ENDOTHELIUM IN THYROID DYSFUNCTIONS

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Abbreviations

AbTg: anti-thyroglobulin antibodies

ADMA: asymmetric dimethyl arginine

AKT : phosphatidylinositol 3-kinase (PI3K)/ serine/threonine-protein kinase

BMI: body mass index

COX-2: cyclooxygenase 2

CFR: Coronary flow reserve

DBP: diastolic blood pressure

DTC: differentiated thyroid cancer

eNOS: endothelial NO synthase

FMD: Flow-Mediated Vasodilation

FT3: free Triiodotironine

FT4: free Thyroxine

L-NMMA: NG_monomethy-L arginine

NO : nitric oxide

PAF: platelet activating factor

PLN: phospholamban

rhTSH: recombinant human TSH

SBP: systolic blood pressure

SERCA2: sarcoplasmic/endoplasmic reticulum calcium ATPase 2

SubHypo: subclinical hypothyroidism

SubHyper: subclinical hyperthyroidism

T3: Triiodotironine

T4: Thyroxine

Tg: thyreoglobulin

TRAIL: TNF-related apoptosis-inducing ligand

TSH: Thyrotropin

1. Introduction

The relationship between thyroid hormones and cardiovascular system has been extensively demonstrated in experimental and clinical studies (1-4). This association has been confirmed by significant changes in cardiac structure and function in patients with overt or subclinical thyroid dysfunctions (1-11). The endothelium and vascular smooth-muscle cells are biological target of thyroid hormones action and their role have been investigated in hypothyroidism and hyperthyroidism reporting different results (1-3, 5, 6).

1.1 Thyroid Hormones actions on the heart

Thyroid hormone has many effects on the heart and vascular system *via* genomic and non-genomic effects, affecting chronotropic, inotropic and lusitropic functions (1-3).

Fig 1

Triiodotironine (T3), the biological active thyroid hormone, enters into the cardiomyocytes through cell membrane transporters and is also produced by the intracellular conversion of Thyroxine (T4) by type II deiodinase. T3 binds thyroid hormone nuclear receptor with the activation or repression of genes transcription for structural and functional cardiac proteins. T3 activates gene expression encoding Na/K-transporting ATPases, myosin heavy chain-α (myosin 6, important component of the cardiomyocyte contractile apparatus) and sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2). Contrarily, T3 down-regulates the transcription of myosin heavy chain-β (myosin 7) and phospholamban (PLN). SERCA2 and its

inhibitor PLN regulate the intracellular calcium concentration, an important determinant of both cardiac relaxation and contraction, by acting on calcium reuptake and release from the sarcoplasmic reticulum. Thyroid hormones increase SERCA2 levels and decrease PLN levels promoting cardiomyocytes calcium reuptake during diastole, which is the major determinant of the myocardial velocity relaxation after contraction (1-3). Thyroid hormones trigger the gene transcriptional activation encoding the β1-adrenergic receptor with a direct inotropic effect on the heart (3).

In addition to these genomic effects, thyroid hormones produce changes in cardiac inotropism and chronotropism that are not mediated by the interaction between thyroid hormones and their nuclear receptors. These effects, known as non-genomic effects, determine ion membrane channel activation (sodium, potassium and calcium), activation of phosphatidylinositol 3-kinase (PI3K)/ serine/threonine-protein kinase (AKT) resulting in an inhibitory effect of PLN on sarcoplasmic reticulum calcium-activated ATPase.

The endothelium and the smooth muscle cells are a biological target of action for thyroid hormones with a vasodilator effect. Thyroid hormone acutely decreases peripheral vascular resistance by increasing calcium reuptake within the arterioles with subsequent smooth muscle relaxation. T3 improves systemic vascular resistance by both endothelium-dependent and independent mechanisms (2, 3). Thyroid hormone stimulates the production of endothelial nitric oxide with a subsequent reduction in the systemic vascular resistance (1-3). Moreover, T3 determinates also vasodilation and changes in the renin–angiotensin–aldosterone system by reducing kidneys perfusion. Higher renin and aldosterone levels increase plasma volume and cardiac preload (3).

1.2 The endothelium

The endothelium, once considered only a passive interface between blood and vessel walls, is now known to be a homeostatic organ with endocrine and paracrine functions. It is essential for the regulation of the vascular tone and structure (12). Endothelial cells, in fact, are able to synthesize and secrete anti-atherosclerotic substances: nitric oxide (NO), endothelium derived hyperpolarizing factor, platelet activating factor (PAF), endothelin1prostaciclin, tromboxan A2 and prostaglandine A2. The most important of these, NO, is a gas produced through a 5-electron oxidation of the guanidine-nitrogen terminal of L-arginine *via* endothelial NO synthase (eNOS), expressed in endothelial cells, in response to shear stress elicited by circulating blood (13, 14). NO diffuses to surrounding tissue and cells exerting a cardiovascular protective role by relaxing media-smooth muscle cells, preventing leukocyte adhesion and migration into the arterial wall, muscle cell proliferation, platelet adhesion and aggregation, and adhesion molecule expression (15, 16). An intact endothelial layer is crucial in preventing circulating blood cell exposure to prothrombotic subendothelial matrix, in avoiding platelet and leukocyte interaction with the vascular wall and in inhibiting vascular smooth muscle cell proliferation and migration (17, 18). Moreover, a healthy endothelium inhibits arterial thrombus formation. Indeed, NO limits platelet activation, adhesion and aggregation, and inhibits the expression of prothrombotic protein plasminogen activator inhibitor-1 modulating the balance of profibrinolytic and prothrombotic activity (19). Furthermore, platelets have been shown to express eNOS and to produce NO that limits platelet recruitment and aggregation. Endothelial

prostacyclin production, largely dependent on cyclooxygenase 2 (COX-2), acts synergistically with NO to prevent platelet activation (20) Fig.2.

The NO bioavailability represents the main key marker regarding vascular health (12, 14). Under pathologic conditions, in fact, the endothelium reduces the availability of vasodilating factors, in particular NO, and augments the production of vasoconstricting factors, leading to impaired endothelium-dependent vasodilation (12).

Endothelial dysfunction is a pathological condition characterized by an imbalance between substances with vasodilating and antithrombogenic properties and substances with vasoconstricting, prothrombotic and proliferative characteristics (16, 21). Endothelial dysfunction is involved in the early steps of atherosclerosis development (increased lipid permeability and the promotion of oxidative and inflammatory environments for atheromatous plaque creation, progression and evolution in necrotic plaques particularly vulnerable to rupture) and is associated with an increased risk of cardiovascular events (12, 22).

Presently, no techniques have been developed to allow a direct visualization of the coronary microcirculation in vivo in humans. To asses coronary endothelial function several invasive and non-invasive techniques are performed to measure parameters that are strongly dependent on the functional integrity of the coronary microcirculation (23, 24). Table 1

Despite its key importance in vascular pathophysiology, NO can be hardly assayed directly due to its biochemical properties; NO, in fact, is a volatile gas with a short half-life (6-7 sec). For this reason, several invasive and non-invasive indirect methods for assessing stimulated NO release both in coronary and peripheral vascular districts have been developed (14, 23). Endothelial function is usually tested analyzing the

degree of change in vessel diameter both in the microcirculation (coronary, peripheral muscle, subcutaneous and skin circulation) and in the macrocirculation (epicardial or brachial arteries) (23, 24).

Changes in coronary blood flow can be used as a surrogate parameter for microvascular function (21, 25). Coronary flow reserve (CFR) represents the capacity of the coronary circulation to dilate following an increase in myocardial demands. It could be measured as the ratio between maximal coronary hyperemia with provocative stimuli (physical or pharmacological) and coronary flow at rest (26, 27). In normal conditions and in the absence of the stenosis coronary blood flow increases approximately about 4-6 times as a consequence to the myocardial oxygen consumption. This effect, mediated by the arteriolar vasodilatation, causes a reduction of vascular resistances and an increase of the coronary blood flow (26). The assessment of CFR by trans-thoracic Doppler method has been established as a reliable, inexpensive, non-invasive and easily executable method in clinical practice for the evaluation of coronary blood flow at rest and after pharmacological (dipyridamole or adenosine) or physical stimulation (Cold pressure test (CPT) (23, 28). Other methods to evaluate the CFR such as the thermal dilution in the coronary sinus, the cardiac nuclear magnetic resonance, positron emission tomography and intracoronary Doppler flow wire are rarely used in clinical practice because of high costs, high time-consuming, lack of availability and invasiveness (23, 28).

Quantitative coronary angiography or intravascular ultrasound are methods to evaluate epicardial coronary arteries endothelium (23, 26). After pharmacological (acetylcholine and salbutamol intracoronary infusion) or physical stimulation (CPT or exercise) vessels with an integral endothelium are able to vasodilate with a consequent

increase in coronary blood flow. On the contrary in presence of dysfunctional or disrupted endothelium there is a decrease in coronary blood flow; acetylcholine determinates a vasoconstriction effect on the vascular smooth cells in absence of NO release. Although these methods are invasive, expensive, their advantage is to measure endothelial function directly in the coronary vascular bed (16).

Flow-Mediated Vasodilation (FMD) of Brachial Artery is a non-invasive method, based on the measurement of percentage change in brachial artery diameters from baseline and after an increase in shear stress induced by reactive hyperemia. This technique measures the ability of the arteries to respond with endothelial NO release following a brief period of reactive hyperemia induced by brachial artery occlusion using a blood pressure cuff. The peripheral endothelial function assessed by FMD correlates with coronary artery endothelial function (16, 29).

Plethysmography of the forearm circulation is a semi-invasive technique to measure changes in forearm blood flow before and after vasoactive substance infusion (vasodilator substances as acetylcholine and nitroglycerin or vasoconstrictor substances as noradrenalin and L-NMMA). The variations in forearm blood circulation are evaluated by use of a cannulated brachial artery that evaluates venous plethysmography (16).

Measuring endothelial function with peripheral arterial tonometry has been developed to quantify observer independent pulsatile arterial volume changes by finger plethysmography (EndoPAT) (16). The pulse amplitude increase after reactive hyperemia reflects changes in digital microvessel flow and dilatation, only partly dependent on NO. The endothelial function alterations in peripheral finger measured

by EndoPAT were correlated with coronary microvascular function in patients with early atherosclerosis and could predict cardiovascular events (30).

The correlation between endothelial dysfunction and cardiovascular risk factors has been evaluated in several studies. Endothelial dysfunction has been associated with hypertension, diabetes mellitus, dyslipidemia, age, hyperhomocysteinemia, smoking, and obesity (31-35). A progressive impairment in NO availability has been showed in the presence of multiple risk factors, with a probable additive effect on the endothelium (36).

Endothelial dysfunction has been also analyzed in those patients with thyroid dysfunctions.

1.3 Endothelial dysfunction in overt and subclinical Hypothyroidism

Hypothyroidism is a clinical condition characterized by elevated serum Thyrotropin (TSH) and low thyroid hormones, while Subclinical Hypothyroidism (SubHypo) represents a condition characterized by elevated serum TSH and thyroid hormone levels within their respective reference ranges. Dependent on the degree of serum TSH increase, SubHypo is divided in two forms: mild (serum TSH increase 4.5–9.9 mU/L) or severe (TSH ≥10 mU/L) thyroid hormone deficiency (5, 6).

Overt and subclinical hypothyroidism are associated with endothelial dysfunction (1-7).

Hypothyroidism impairs endothelial function by the reduction of NO availability, relaxation of vascular smooth muscle cells with increase in systemic vascular resistance and arterial stiffness (1-3, 11, 37-44).

Hypothyroidism is associated with microvascular endothelial dysfunction in women. The evaluation of endothelial-dependent microvascular and epicardial function in coronary artery in response to intracoronary infusions of acetylcholine, showed that hypothyroid women presented a microvascular endothelial dysfunction compared to euthyroid women. This alteration did not change after adjusting for confounders and risk factors and may explain the increased risk of coronary heart disease in these patients (37).

Flow mediated endothelium-dependent vasodilatation, assessed by high-resolution ultrasound imaging of the brachial artery, was significantly impaired in hypothyroid subjects with SubHypo (TSH levels between 4.01 and 10 mIU/liter, and greater than 10 mIU/liter) and in subjects with "high-normal" serum TSH levels (TSH: 2.01-4.0 microIU/mL) compared to a control group (38).

The negative effects of SubHypo on cardiovascular function could be improved or reversed by replacement doses of levothyroxine (LT4). Randomized controlled trials showed that LT4 treatment improved endothelial function, systolic and diastolic function and carotid intima-media thickness in patients with SubHypo (5, 6, 39-43).

Taddei et al. analyzed the FMD response to intrabrachial acetylcholine infusion at baseline and during infusion of a NO synthase inhibitor (NG-monomethyl-l-arginine - L-NMMA) in young, non-obese, non-smokers and non-hypertensive SubHypo patients before and after LT4 therapy (39). The authors evaluated the response to sodium nitroprusside and minimal forearm vascular resistances too. In SubHypo patients, vasodilation to acetylcholine was reduced compared to euthyroid subjects, while the response to sodium nitroprusside (which specifically acts on vascular smooth muscle cells) was similar in patient with or without SubHypo. Patients with SHypo had

endothelial dysfunction with a reduction in NO availability; this alteration was partially independent of dyslipidemia and reversed after six months of LT4 supplementation (39).

In a subsequent randomized crossover trial, FMD improved significantly in patients with SubHypo after replacement LT4 therapy, independently of other cardiovascular risk factors (40). The enhancement in FMD response could suggest a better NO production and an improvement in endothelial function in clinically relevant areas of the vasculature, such as coronary and carotid circulation (40).

In a double-blind placebo controlled study a reduction in carotid artery intima-media thickness (an atherosclerosis parameter) after replacement therapy with LT4 in patient with SubHypo was also reported. This reduction was directly related to the decrease of both total cholesterol and TSH values after therapy (41).

Numerous factors could potentially contribute to arterial stiffness and endothelial dysfunction in SubHypo as in overt hypothyroidism. Hyperlipidemia has an important role in atherosclerosis development (41). SubHypo patients may have higher Triglycerides, LDL-Cholesterol and apolipoprotein B levels and higher mean intima-media thickness values, compared with euthyroid patients matched fox sex and age (40). Thyroid autoantibodies might also have a potential role in inflammatory processes associated to endothelial dysfunction. In patients with SubHypo due to thyroid autoimmunity, the endothelial dysfunction after LT4 therapy was only partially reversed (41). Low-grade chronic inflammation due to Hashimoto's thyroiditis (an autoimmune thyroid disease) induced an impaired NO availability by COX-2-dependent pathway in patients with SubHypo (41). Both hyperlipidemia and thyroid

antibodies are able to reduce the eNOS expression impairing NO production and endothelium-dependent vasomotor function (41).

Furthermore, thyroid hormone deficiency reduces NO availability in the coronary endothelium of mild SubHypo patients without associated cardiovascular risk factors (44).

Thyroid hormone exerts an important effects on the endothelial cell through the thyroid hormone receptor α1 and thyroid hormone receptor β. The activation of thyroid hormone receptor α1 improved myocardial perfusion, reduced coronary resistance in transgenic mouse models (45) and activated of PI3K/AKT signaling and NO synthase in endothelial and vascular smooth muscle cells in rat thoracic aortas (46). Thyroid hormone binds thyroid hormone receptor β activating the mitogen-activated protein kinase (MAPK) pathway with the activation of proangiogenic genes transcription (genes encoding: vascular endothelial growth factor (VEGF), basic fibroblast growth factor and angiopoietin) (47, 48).

Previous studies on circulating markers of endothelial dysfunction have been performed (49-51). Tejovathi et al. demonstrated that hypothyroid patients presented higher asymmetric dimethyl arginine (ADMA) levels and lower plasma nitrate levels compared to euthyroid patients, confirming the presence of endothelial dysfunction in hypothyroid patients (49). Xiang et al. investigated the relationship between TNF-related apoptosis-inducing ligand (TRAIL) and endothelial dysfunction in SubHypo patients. They reported that the decreased TRAIL levels in SubHypo patients, were positively associated with endothelial function (50). Gungor et al. showed elevated endothelial cell-specific molecule-1 (endocan) levels in overt hypothyroid patients,

suggesting that endocan levels may be an early biomarker of the development of endothelial dysfunction in hypothyroid patients (51).

In conclusion, based on the data available, overt and subclinical hypothyroidism could impair vascular function by altering endothelial function, thus potentially increasing the risk of atherosclerosis and coronary artery disease.

1.4 Endothelial dysfunction in overt and subclinical Hyperthyrodism

Hyperthyroidism is characterized by a suppressed serum TSH with elevated thyroid hormone levels. Subclinical Hyperthyroidism (SubHyper) represents a condition characterized by low or undetectable serum TSH concentration with free T4 and T3 concentrations within their normal ranges. SubHyper can be divided in two forms: endogenous SubHyper, caused by Graves' disease, autonomously functioning thyroid nodules or toxic multinodular goiter, and exogenous SHyper, secondary to an intentional TSH suppression during L-Thyroxine therapy (5, 6).

Overt hyperthyroidism is characterized by hyperdynamic cardiovascular function, with high cardiac output, improved diastolic function and low systemic vascular resistance with a compromised left ventricular performance during effort (1-4).

SubHyper, may induce changes in cardiac morphology (increased left ventricular mass) and function (impaired diastolic filling and exercise tolerance) and be responsible for alteration in cardiac rhythm (sinus tachycardia, atrial fibrillation, increase in atrial and ventricular premature beats) (2, 5, 6, 11). Thyroid hormone excess acts on the endothelial component of vascular reactivity, with an NO increase production. Napoli et al. observed an increase in NO production with an elevate

forearm blood flow in basal state and after acetylcholine infusion in untreated hyperthyroid patients with Graves' disease. These alterations normalized after methimazole therapy when euthyroidism was reached (52).

SubHyper represents a negative prognostic factor for cardiovascular mortality and morbidity in the general population (8, 9, 53-55). An increase arterial stiffness and a higher carotid intima-media thickness were observed in patients with long term SubHyper compared with euthyroid and hypothyroid patients (55). A meta-analysis of large prospective studies of patients with endogenous or exogenous persistent SubHyper demonstrated that low serum TSH levels was associated with an increased risk of coronary heart disease mortality and all causes of mortality, with highest risks in presence of undetectable TSH levels (<0.1 mU/l) (53)

1.5 *Endothelial dysfunction and TSH*

The thyroid hormones role on the heart and the cardiovascular system has been extensively analyzed in patients with subclinical and overt thyroid dysfunctions. On the contrary, the potential pathophysiological role of the TSH receptor in the cardiovascular system is unclear because it has been poorly investigated in vivo. In fact, only a few studies have assessed the expression of the TSH receptor in the cardiovascular system in humans (56-58), mainly because of the difficulty in separating its cardiovascular effects from those exerted by thyroid hormones in patients with thyroid dysfunction.

It is unknown, whether the changes in the vasculature associated with thyroid diseases may reflect the expression of the direct effect of TSH levels on endothelial cells.

The presence of TSH receptor has been reported in vitro in extrathyroid tissues such as adipose tissue, smooth muscle cells, red blood cells, hepatocytes and also in endothelial cells from umbilical cord and human aortic tissue (59-63). Sellitti et al. also observed that TSH receptor mRNA expression was highest in the coronary artery of pig heart tissue (60). Donnini et al. reported that TSH increased cAMP and NO production in human aortic endothelial cells (62). In the heart, the TSH receptor has been found in ventricular myocytes (63). However, the pathophysiological role of TSH stimulation through its receptor outside of the thyroid tissue is still unknown.

The studies conducted so far to evaluate the TSH direct effect in the peripheral vascular endothelium in thyroidectomized patients produced conflicting results (56-58). Moreover, the effect of TSH on coronary endothelial cells has never been directly studied in humans before our study (64).

2. Aim of the study

The aim of our study was to investigate the endothelial response of coronary flow to recombinant human TSH (rhTSH) in disease-free patients with differentiated thyroid cancer (DTC) without cardiovascular risk factors to assess the direct effects of TSH on coronary endothelium (64).

This study was of particular interest for several reasons:

- the direct role of TSH on the coronary endothelial cells has never been directly studied;
- we used a non-invasive method (the evaluation of CFR by trans-thoracic eco Doppler) and a physical stressor (CPT);
- we analyzed thyroidectomized patients with low-risk DTC that represent an ideal experimental model to assess the acute TSH effects on the cardiovascular function. In fact, low-risk DTC patients require replacement doses of L-T4 (65, 66). L-thyroxine (LT4) enables these patients to maintain euthyroidism, so that one can evaluate the effects of serum TSH without stimulating TSH-dependent thyroid hormone secretion.

*3. **Methods***

*3.1 **Study population***

The study population consisted of 10 consecutive patients (3 men and 7 women, mean age: 32.6±8 years) who were submitted to total thyroidectomy for DTC in follow up at our outpatient thyroid cancer clinic at the "Federico II University Hospital" of Naples. The study protocol was approved by the Ethics Committee of the "Federico II University Hospital" of Naples and all patients provided informed consent to the study. All enrolled patients had a low-risk DTC according to the American Thyroid Association Guidelines (absence of local or distant metastases, resection of all macroscopic tumor, absence of tumor invasion of locoregional tissues or structures, not aggressive histology and, if ^{131}I were given, no ^{131}I uptake outside the thyroid bed on the first post-treatment whole-body scan) (65). No patient had anti-thyroglobulin antibodies (AbTg). Low-risk DTC patients were receiving LT4 doses to maintain TSH within normal range (mean dose: 1.97μg/kg/die; range: 1.68-2.16 μg/kg/die). We carried out a strict patient selection to exclude all the factors that could confound the evaluation of the CFR (47, 64, 67-69).

Exclusion criteria were pregnancy, obesity (body mass index $\geq$ 30 kg/m^2), arterial systemic hypertension, diabetes mellitus, insulin resistance, dyslipidemia, heart disease, cardiac rhythm abnormalities, hepatic or renal disorders, cigarette smoking, a personal or family history of coronary artery disease and the assumption of any kind of medication apart from L-T4. Dyslipidemia was defined as total cholesterol $\geq$ 200 mg/dl and/or triglycerides $\geq$ 160 mg/dl; and arterial hypertension was defined

as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg. Insulin resistance index, HOMA, was assessed with the homeostasis model of insulin resistance (insulinemia [mU/L] × glycemia [mmol/L])/22.5 (27). Coronary artery disease was excluded based on clinical history and effort ECG. Hepatic and renal diseases were excluded on the basis of the history and laboratory tests (transaminases and creatinine) (64).

3.2 ***Study protocol***

This study was performed during the periodical post-surgical follow-up of our DTC patients after the administration of recombinant human TSH (rhTSH) to assess stimulated Tg levels. The aim of the DTC post-surgical follow-up is an early detection and treatment of persistent or recurrent loco regional or distant disease. Therefore follow-up is based on periodic evaluation of basal and stimulated thyroglobulin (Tg) after rhTSH administration and neck ultrasound (65). rhTSH is a heterodimeric glycoprotein produced by recombinant DNA technology. The rhTSH amino acid sequence is identical to the human pituitary TSH and presents the same biochemical proprieties. rhTSH has been proposed as an alternative to LT4 withdrawal in the staging of patients with DTC (70).

Patients received two intramuscular injections of rhTSH, at a dose of 0.9 mg each, on two consecutive days. Serum TSH, thyroid hormones, Tg and anti-thyglobulin antibodies (AbTg), metabolic parameters (weight, height, body mass index (BMI), glycemia, total cholesterol and triglycerides) were determined at baseline. Serum TSH, thyroid hormones and Tg were re-evaluated 24 hours after the second injection of

rhTSH (70). Replacement doses of LT4 were unchanged throughout the study. Patients underwent standard Doppler echocardiography with the evaluation of CFR of the left anterior descending artery by the CPT before and 24 hours after the second rhTSH injection (64).

3.3 *Assessment of thyroid status*

Blood samples for circulating serum TSH, free triiodothyronine (FT3), free thyroxin (FT4), Tg and AbTg were collected from the antecubital vein between 7.00 and 8.00 hours after an overnight fast. TSH levels were measured with the electrochemiluminescence immunoassay (ECLIA; Elecsys and Cobas analyzers, Roche Diagnostics) that has a sensitivity of at least 0.005 mIU/l, and the reference range was 0.3-4.2 mIU/l. Serum FT3 and FT4 were also measured with the ECLIA. Sensitivities were <0.400 pmol/l and <0.300 pmol/l for FT3 and FT4, respectively. The reference ranges were 3.1-6.8 pmol/l for FT3, and 12-22 pmol/l fo FT4. Serum Tg levels were also measured with ECLIA with a sensitivity of 0.1 ng/ml or less, and the reference range was 0.1-50 ng/ml. Serum AbTg levels were measured with ECLIA and the reference range was 0-115 IU/ml (64).

3.4 Standard echocardiographic assessment

Echo-Doppler examinations were performed with a Vivid Seven Sound machine (GE, Horten, Norway) equipped with a 2.5 MHz phased-array transducer with harmonic capability.

M-Mode tracings were recorded in parasternallong-axis view, and left ventricle, left atrium and aortic root sizes were measured as previously reported (68). Left ventricular mass was calculated according to the American Society of Echocardiography recommendations and normalized for height in meters powered to 2.7 using Simpson method (71). Left ventricular end-diastolic and end-systolic volume were measured in apical 4- and 2-chamber views and left ventricular ejection fraction calculated according to the standard formula. Standard pulsed Doppler imaging of mitral inflow was recorded in the apical four-chamber view. Early (E) and atrial (A) peak velocities (m/s) and their ratio, E velocity deceleration time (ms) and isovolumetric relaxation time (ms) were measured. Pulsed tissue Doppler-derived early diastolic velocity (e') of septal and mitral annulus were measured and averaged. The E/e' ratio was derived as a non-invasive estimate of left ventricular filling pressure. Echo-Doppler methods and the reproducibility of our laboratory are reported elsewhere (26, 27). All images were analyzed off-line by two observers who were blind to the patients' clinical characteristics (64).

3.5 Coronary flow reserve

Coronary flow was visualized in the distal left anterior descending artery by transthoracic Doppler echocardiography with a 5 MHz shallow-focus phased-array transducer. Doppler sample volume was placed on the color signal of the left anterior descending artery, and the spectral pulsed Doppler signal was recorded to look for the characteristic biphasic flow pattern with a larger diastolic and a smaller systolic component both at rest and after the CPT. Coronary diastolic peak flow velocities

(cm/s), heart rate and blood pressure were measured at rest and soon after the CPT at maximal endothelial induced hyperemia. The coronary flow reserve was calculated as the ratio of hyperemic-to-resting diastolic peak velocities. The CPT was performed according to our standardized protocol, by placing the subject's hand and distal part of the forearm in an ice water slurry for 4 minutes (19, 67). The highest three spectral Doppler signals were averaged for each parameter. The reproducibility of our CPT-derived coronary flow reserve measurements of our echo laboratory are: an intra-observer variability of 2.0% and an inter-observer variability of 4.5%, as previously reported (26). All images were analyzed off-line by two observers who were blinded to the clinical characteristics of the patients. All examinations were performed between 8.00 and 9.00 hours. All patients were fasting and abstained from coffee for at least 12 h before testing (64).

3.6 Statistical analysis

SPSS for Window (version 20) was used for statistical analyses. All data are presented as mean values ± SD, approximated at the second decimals. The ANOVA test was used to assess intergroup differences. Linear regression analyses and the partial correlation test using Pearson's correlation method were used to test univariate relations. Differences were considered statistically significant at $p<0.05$ (64).

*4. **Results***

The clinical, demographic and anthropometric characteristics of the patients at baseline (before the first injection of rhTSH) are shown in Table 2 (64).

Eighty consecutive patients were analyzed in our ambulatory. Of these, 46 presented one or more exclusion criteria (hypertension, diabetes mellitus, obesity, dyslipidemia, heart disease, cardiac rhythm abnormalities, hepatic or renal disorders, cigarette smoking, personal or family history of coronary artery disease). 34 patients were assessed for eligibility. Five were excluded, during the enrolment, following the development of systemic disorders with consequent assumption of medications. 7 patients were excluded because of elevated BMI (BMI>27 kg/m²) and insulin resistance. 2 women became pregnant. 10 patients were eliminated from the study after the first cardiological evaluation; of these, 6 patients were excluded for a poor acoustic windows and 4 because they refused the second echocardiographic evaluation. 10 patients which presented all the inclusion criteria were enrolled in this study. Fig. 3

All of the patients included in the study presented a TMN staging of T1N0M0 and had a low risk of tumor recurrence during the follow-up, according to the American Thyroid Guidelines (65).

In accordance with the exclusion criteria no patient enrolled presented obesity (BMI 24.51 ± 2.46 kg/m² [data are expressed as means ± SD]), hypertension (SBP 115±14.1 and DPB 68.5±9.4 mmHg), dyslipidemia (total cholesterol 177.9±16.5 mg/dl and triglycerides 66.2±16.8 mg/dl) or hyperglycemia (84.8±6.7 mg/dl). As a consequence of replacement doses of L-T4, the TSH level at baseline was 1.25 ± 0.37 μU/ml and serum FT3 and FT4 were within the normal range. No patient had AbTg or

elevated Tg values. The left ventricular structure, and systolic and diastolic function at echo-Doppler analysis were normal in all patients (64) Table 3.

Table 4 shows the results of the CFR at baseline and at 24 h after second rhTSH injection (64). Mild side effects, such as nausea and headaches, never occurred during rhTSH administration. TSH levels were significantly higher than baseline after rhTSH administration (147±37 vs 0.7±0.3; p <0.001), whereas thyroid hormones, FT3 and FT4, Tg and AbTg levels remained unchanged (64). Similarly, rhTSH administration did not affect SBP (115±14.1 at baseline vs 119±13.1 after rhTSH), DBP (68.5± 9.4 vs 73±9.7), mean blood pressure (85±9.9 vs 83±6,4) or heart rate (75.9±12.9 vs 74.6±12.2). Coronary flow peak velocity at rest was similar at baseline and 24 hours post-rhTSH (22.3 ± 6 vs 23.2 ± 8.7; p = 0.66), whereas coronary flow peak velocity after CPT was higher after rhTSH administration (29.3 ± 6.8 vs 34.4 ± 10.9; p<0.05). Of consequence, CFR was significantly higher after administration of rhTSH (1.3 ± 0:2 vs. 1.5 ± 0.2, p <0.01). The increase in myocardial work, reflected by the increase in the rate pressure product (SBP x heart rate), was significantly higher after rhTSH administration (8887.1 ± 2883.4 vs 9880.7 ± 2717.9, p<0.05), and was associated with a proportionate increase in myocardial blood flow (64).

Figure 4 shows the CPT-derived coronary flow at baseline and 24 hours after the second rhTSH injection in one patient (64). Figure 5 shows changes in coronary flow velocities after the cold pressure test at baseline and 24 h after the second rhTSH administration in each patient (64). We found that coronary flow improved after rhTSH administration in 8/10 patients (80%). The CFR 24 hours after the second rhTSH injection was not significantly related to heart rate (p=0.4), SBP (p=0.6),DPB (p=0.5), left ventricular mass (p=0.8), relative wall thickness (p=0.5), indices of left

ventricular diastolic function (a peak velocity p=0.7; e peak velocity p=0.7; e/a ratio p=0.7), systolic function (p=0.1), fasting blood glucose (p=0.6), total cholesterol (p=0.8), triglycerides (p= .0.6), BMI (p=0.2) or TSH (p=0.7) (64).

5. *Discussion*

The present study evaluates the effect of TSH on the coronary endothelial function in athyreotic patients without cardiovascular risk factors during treatment with replacement doses of LT4 (64). All the patients enrolled were young, non-obese, non-smokers with normal blood pressure, normal cholesterol levels and absence of a personal or family history of coronary artery disease. The administration of rhTSH did not change heart rate, cardiac rhythm, left ventricular structure or systolic and diastolic function in our thyroidectomized patients, as previous reported (72). Moreover, the non-pharmacological stressor CPT improved the CFR after rhTSH administration in DTC patients (64).

The evaluation of rhTSH effects on the endothelium have been reported conflicting results (56-58). Napoli et al. analyzed the positive effects of TSH in vascular homeostasis in large conduit arteries and small resistance vessels using forearm blood flow dilatation measured by plethysmography (56,57). Acute intrabrachial administration of rhTSH enhanced endothelium-dependent vasodilatation (evaluated during acetylcholine infusion) but not endothelium-independent vasodilatation (evaluated during nitroglycerine administration) in thyroidectomized patients and health volunteers. Acute rhTSH administration improved endothelial function in the small resistance vessels, irrespective of thyroid hormone secretion (56). Similarly, in another study, the authors evaluated the role of TSH on large arteries in thyroidectomized patients with DTC using FMD of the brachial artery. They reported a marked and persistent activation of the endothelial-mediated vasodilatation persisted for 5 days after the rhTSH administration, independent of systemic hemodynamic changes (57). The results of this study supported

that the acute administration of rhTSH is able to enhance vascular reactivity in the human conduit arteries. This response was mediated by endothelial mechanisms as witnessed by the finding that rhTSH did not alter the response to nitroglycerin, which is a vasorelaxing agent that acts directly on smooth muscle cells (57, 64).

On the contrary, in another study the administration of rhTSH acutely impaired endothelium-dependent vasodilatation possibly by inducing low-grade inflammation in patient with DTC; a sharp rise in serum TSH induced a significant reduction in FMD with an increase in blood inflammatory markers (IL6, TNFα) (56). The discrepancy between these studies may be related to the different methods used to evaluate endothelial function. In fact, FMD analyzes the conduit arteries (58), whereas plethysmography explores resistance vessels (56). Furthermore, one study evaluated athyreotic patients receiving replacement doses of LT4 (56), whereas the other assessed the acute effects of rhTSH in patients receiving TSH suppressive LT4 therapy (58).

In the present study, we demonstrated that CPT improves the CFR after rhTSH administration in DTC patients receiving replacement doses of LT4 (64) (Table 4). The improvement of CFR was mainly determinate to a significant increase of CPT-derived coronary flow. The increase of post-CPT coronary blood flow after rhTSH administration could be related to the direct vascular effect of rhTSH on the coronary endothelial cells and not mediated by thyroid hormones action. The improvement of post-CPT coronary blood flow after rhTSH administration was not associated with a similar increase at rest.

The positive coronary effect could not be considered a reflex response or a consequence of rhTSH action exerted elsewhere in the cardiovascular system because we did not find any cardiac morphology or function alterations, or changes in arterial blood pressure and

heart rate. The absence of AbTg in our study population excludes the possibility of an additional role of inflammation and autoimmunity on the endothelial function (64).

6. Study limitations

The study presents few limitations. One limitation is the small study population size, which may have resulted in the lack of statistical significance of some parameters. However, despite a large standard deviation of rhTSH-derived coronary flow velocity both at rest and after CPT (which demonstrates a degree of overlapping between baseline and rhTSH results), the difference of the same CPT-derived coronary flow was statistically significant, it being increased after rhTSH in 80% of the patients assessed. This result highlights therefore the effect of rhTSH on coronary endothelial function. Nevertheless, it was difficult to enroll young patients with low-risk DTC without cardiovascular risk factors or a family history of coronary disease that were willing to undergo coronary reserve flow evaluation during rhTSH administration (64). The second study limitation is the absence of a well-established cut-off value for CFR evaluated after CPT stimulation. We know that the CPT is less powerful than pharmacological stimulation in NO release by endothelial cells. For these reason the cut-off values can be considered lower (<2), which is the validated cut-off for CFR after pharmacological stimulation (adenosine or dipyridamole). We choose CPT rather than pharmacological stressors because adenosine or dipyridamole determinate a coronary flow increase of which is only partially endothelium-dependent. However CPT stimulation has been validated in previous studies (38, 44) and our results in SubHypo patients supported this lower cut-off (44).

7. Conclusions

Our data demonstrate that acute administration of rhTSH is associated with a significant improvement in the coronary flow reserve. This occurs in response to physiological stimulus induced by CPT as results of increased vascular reactivity mediated by a mechanism of endothelium-mediated vasodilatation. Our results suggest that TSH itself exerts a protective role on the coronary endothelium. This effect could suggest a protective TSH action on coronary endothelium that is direct and not mediated by thyroid hormones modification, because no change in serum FT3 and FT4 levels were observed after rhTSH administration (64). The TSH protective role on the coronary endothelium could explain the negative effects of TSH suppression on cardiovascular disease in patients with SubHyper. Patients with DTC had a higher risk of cardiovascular mortality and low or undetectable TSH levels predicted cardiovascular mortality (73). Therefore, the cardiovascular risk was significantly higher in patients with lower TSH values (53). The mechanism that determinate this amplified vascular risk is unclear and it is in contrast with the positive effects that thyroid hormone excess has on endothelial function and metabolic parameters (52).

The positive TSH effect on the coronary endothelium reported in our study could suggest that SubHyper has negative effects on coronary heart disease determinate by a persistent suppression in TSH, despite the slight increase in thyroid hormone at tissue level levels (64).

8. *Tabels*

Table 1. Advantages and Disadvantages of the Techniques to Assess Endothelial Function

Methods	Vascular Bed	Advantages	Disadvantages	Stimulus
CFR	Coronary microvascular	Non invasive Easy access	Challenging for serial measurements	Dipyridamole Adenosine CPT
Coronary microvascular function by Coronary angiography	Epicardial macrovascular Conduit arteries	Assessment directly in the coronary vascular bed	Invasive Expensive Risks connected to coronary angiography Challenging for serial measurements	Ach Exercise Pacing CPT
Coronary microvascular function–Doppler wires	Coronary microvascular Resistance arteries	Assessment directly in the coronary microvasculature	Invasive Expensive Time intensive Risks connected to coronary angiography Challenging for serial measurements	Ach Adenosine Papaverine
Flow mediated dilatation (FMD)	Brachial artery Conduit artery	Easy access Correlation with invasive epicardial vascular function Inexpensive Possibility to assess other important parameters (flow, baseline arterial diameters, FMC) Directly correlated with coronary endothelial dysfunction	Challenging to perform well Disparate protocols for performance and standardizations Variable measures	Reactive Hyperemia
Plethysmography	Forearm vasculature Microvasculature	Easy access Vasoactive substances infused to generate a dose-response relationship Contralateral arm as a control	Invasive (cannulation of the brachial artery) but less invasive than coronarography Time consuming	Ach and other vasoactive Substances
EndoPAT	Finger Microvasculature	Easy to access and perform automated Low interobserver variability Correlation with invasive microvascular vascular function	Expense of disposable finger probes PAT signal influenced by variable and non-endothelial factors	Reactive hyperemia

Ach, acetylcholine; CPT, cold pressor test; FMC, flow-mediated constriction; PAT, peripheral arterial tonometry

Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, Ganz P, Hamburg NM, Lüscher TF, Shechter M, Taddei S, Vita JA, Lerman A. The assessment of endothelial function: from research into clinical practice. Circulation. 2012; 126(6):753-67. Review (16)

Table 2 Characteristics of the study population*

Clinical data	
Age (years)	32.6 ± 8
Body weight (kg)	69.5 ± 11.4
Height (m)	1.6 ± 0.1
Body mass index (kg/m^2)	24.5 ± 2.4
Hemodynamic characteristics	
Systolic blood pressure (mmHg)	115 ±14.1
Diastolic blood pressure (mmHg)	68.5 ± 9.4
Heart rate (beats/min)	75.9 ± 12.9
Metabolic profile	
Glycemia (mg/dl)	84.8 ± 6.7
Total cholesterol (mg/dl)	177.9 ± 16.5
Triglycerides (mg/dl)	66.2 ± 16.8
Thyroid profile	
TSH (μU/ml)	0.7 ± 0.3
FT3 (pmol/l)	5.2 ± 0.7
FT4 (pmol/l)	19.2± 2.7
Ab Tg (lU/ml)	18.4 ± 14.8
Tg (ng/dl)	0.14 ± 0.21

*Data are expressed as means ± SD

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Table 3 Doppler echocardiography analysis

Variables	Values	Reference range
Left ventricular mass index (g/m²)	67.7 ± 13.7	<95 (W), <115(M)
Relative wall thickness	0.3 ± 0.04	<0.42
Left ventricular ejection fraction (%)	63.7± 6.6	≥53
Left atrial volume index (ml/$m^{2.7}$)	29.9 ± 5.46	<34
Transmitral peak velocity E/A ratio	1.2 ± 0.2	0.73-2.33
E velocity deceleration time (ms)	167.8± 24.5	160-240
E/e' ratio	7.8 ± 1.6	<13

E: transmitral early peak diastolic velocity
A: transmitral atrial peak velocity
e': early diastolic velocity of septal annulus

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Table 4 Coronary flow reserve

Variables	Baseline CFR	CFR after rhTSH	p
TSH (μU/ml)	1.25 ± 0.3	147 ± 37	<0.001
FT3 (pmol/l)	5.2 ± 0.7	5.2 ± 0.2	NS (p=0.17)
FT4 (pmol/l)	19.2± 2.7	19.2 ±2.5	NS (p=0.2)
Ab Tg (lU/ml)	18.4 ± 14.8	18.2 ±14.3	NS (p=0.55)
Tg (ng/dl)	0.14 ± 0.21	0.4 ± 0.35	NS (p=0.08)
Coronary flow velocity at rest (cm/s)	22.3 ± 6	23.2 ± 8.7	NS (p=0.66)
SBP at rest (mmHg)	115 ± 14.1	119 ± 13.1	NS (p=0.3)
DBP at rest (mmHg)	68.5 ± 9.4	73 ± 9.7	NS (p=0.27)
Mean BP at rest (mmHg)	81.6 ± 10.3	82.3 ± 6.5	NS (p=0.85)
Heart rate at rest	75.9 ± 12.9	74.6 ± 12.2	NS (p=0.64)
Coronary flow velocity after CPT (cm/s)	29.3 ± 6.8	34.4 ± 10.9	p <0.05
SBP after CPT (mmHg)	111.4 ± 12.1	115.7 ± 12.3	NS (p=0.17)
DBP after CPT (mmHg)	67.8 ± 5.6	72.1 ± 3.9	NS (p=0.11)
Mean BP after CPT (mmHg)	85.5 ± 7.3	88.3 ± 6.7	NS (p=0.90)
Heart rate after CPT	76.5 ± 16.8	82.7 ± 17.4	NS (p=0.14)
Coronary flow reserve	1.32 ± 0.21	1.53 ± 0.2	p<0.01

Data are expressed as means ± SD
CPT: cold pressure test; SBP: systolic blood pressure; DBP: diastolic blood pressure.

Ippolito S, Ippolito R, Peirce C, Esposito R, Arpaia D, Santoro C, Pontieri G, Cocozza S, Galderisi M, Biondi B. Recombinant human thyrotropin improves endothelial coronary flow reserve in thyroidectomized patients with differentiated thyroid cancer. Thyroid. 2016;26(11):1528-1534 (64)

9. *Figures*

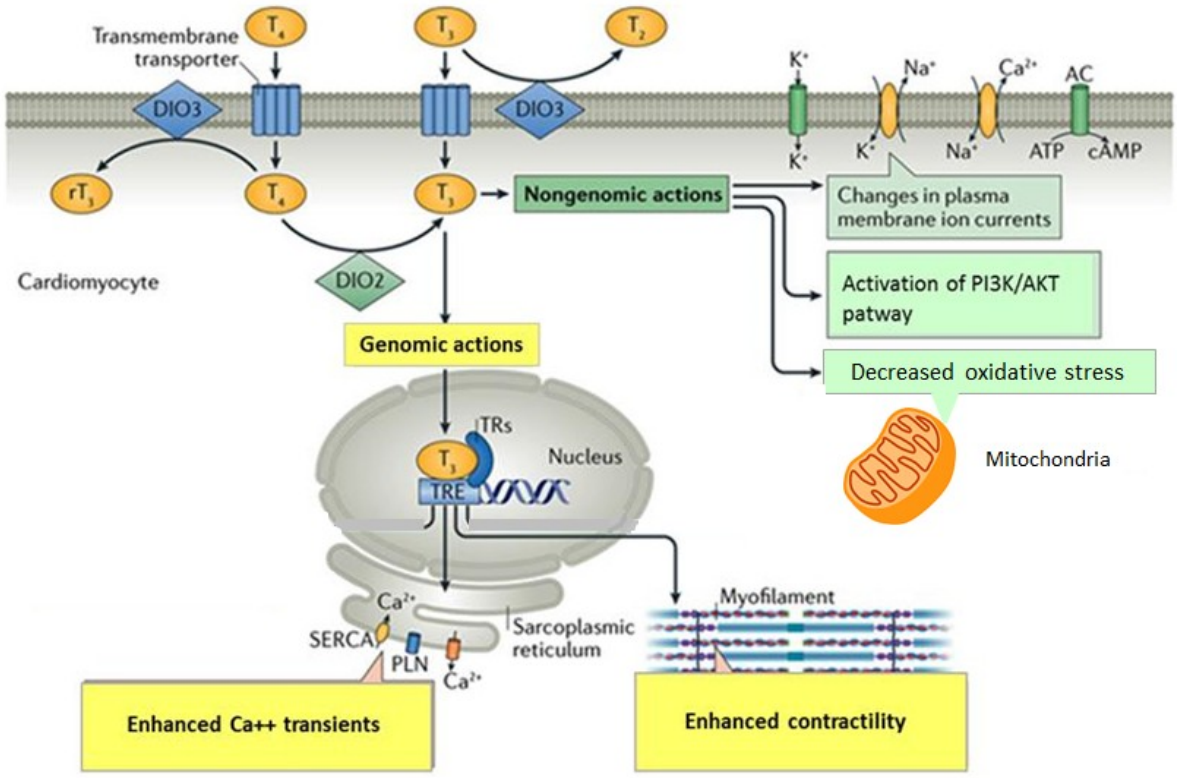

Figure 1 Genomic and nongenomic effect of thyroid hormones on the cardiomyocytes

T3 binds to thyroid hormone receptors (TRs) in the nucleus.
Thyroid hormones positively regulate the transcription of myosin heavy chain-α and sarcoplasmic/endoplasmic reticulum calcium ATPase 2. On the contrary, genes that are negatively regulated by thyroid hormones are those that encode myosin heavy chain-β and phospholamban (PLN).
Nongenomic actions of thyroid hormones include regulation of voltage-gated K+ channels, Na+/K+ ATPase, and the Na+/Ca2+ exchanger, and activation of survival pathways.

T3: Triiodotironine; T4: Thyroxine; AKT:serine/threonine-protein kinase; MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase 2 ; PLN : Phosfolamban

Modified by Jabbar A, Pingitore A, Pearce SH, Zaman A, Iervasi G, Razvi S. Thyroid hormones and cardiovascular disease. Nat Rev Cardiol. 2017; 14(1):39-55. Review. (3)

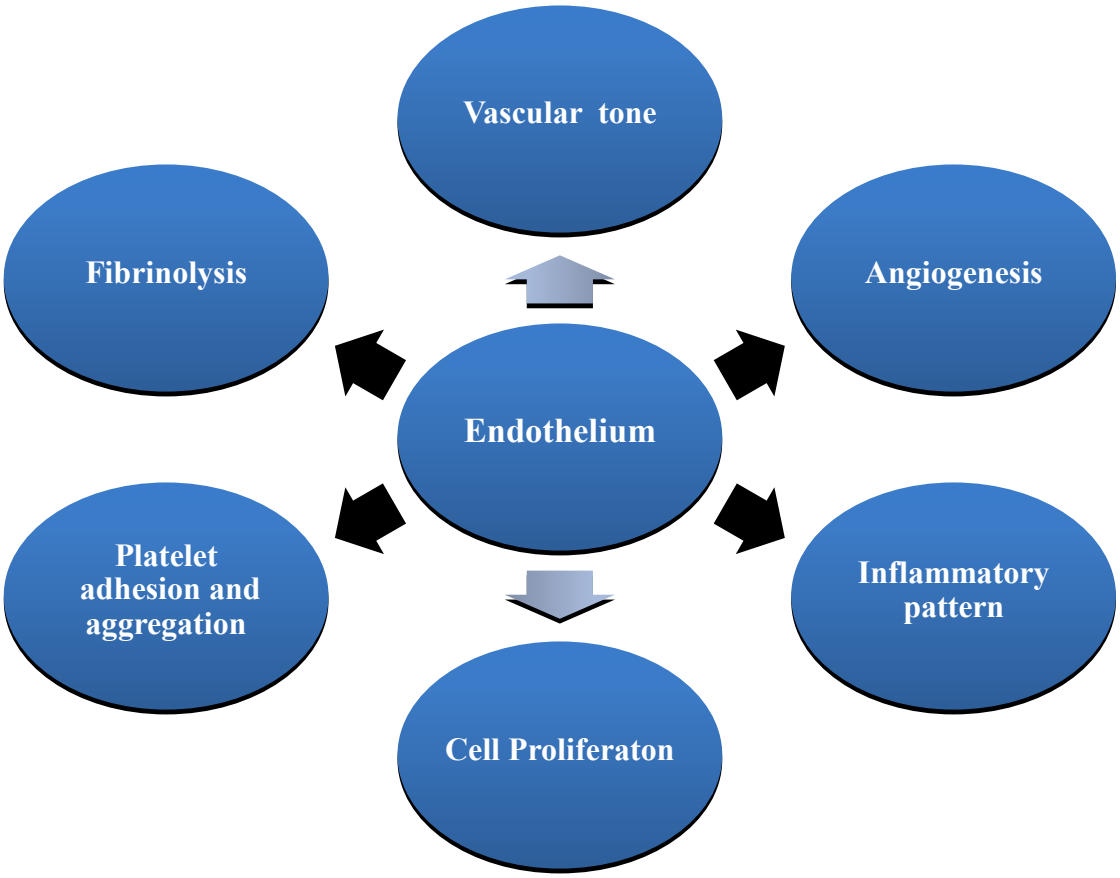

Fig. 2 Multiple endothelial functions

Nitric oxide released from endothelial cells is responsible for a number of physiological functions:
1) regulation of vascular tone through balanced production of vasodilators and vasoconstrictors,
2) control of blood fluidity and coagulation through production of factors that regulate platelet activity, the clotting cascade, and the fibrinolytic system
3) regulation of inflammatory processes through expression of cytokines and adhesion molecules.

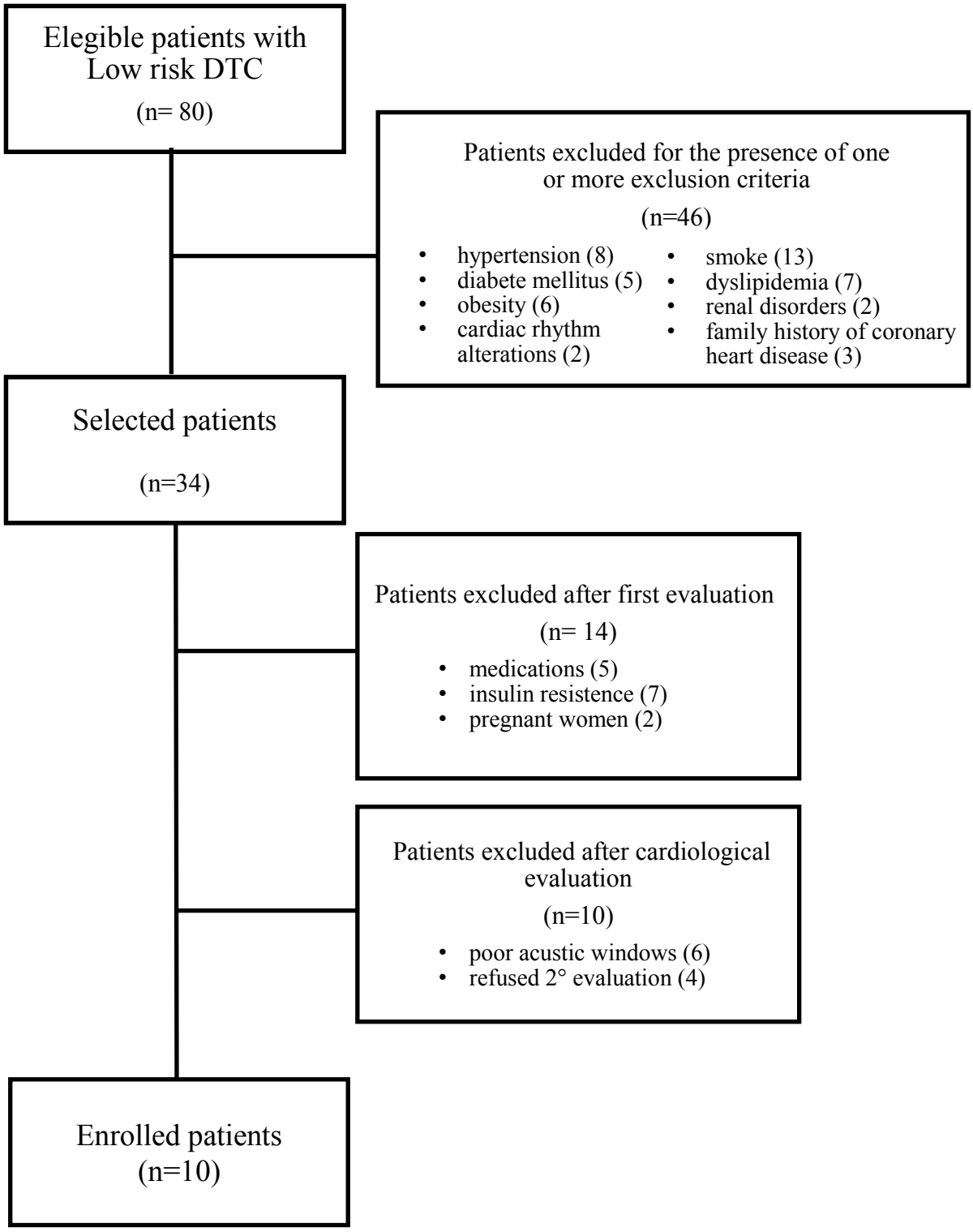

Fig. 3 Flowchart of the patients' selection

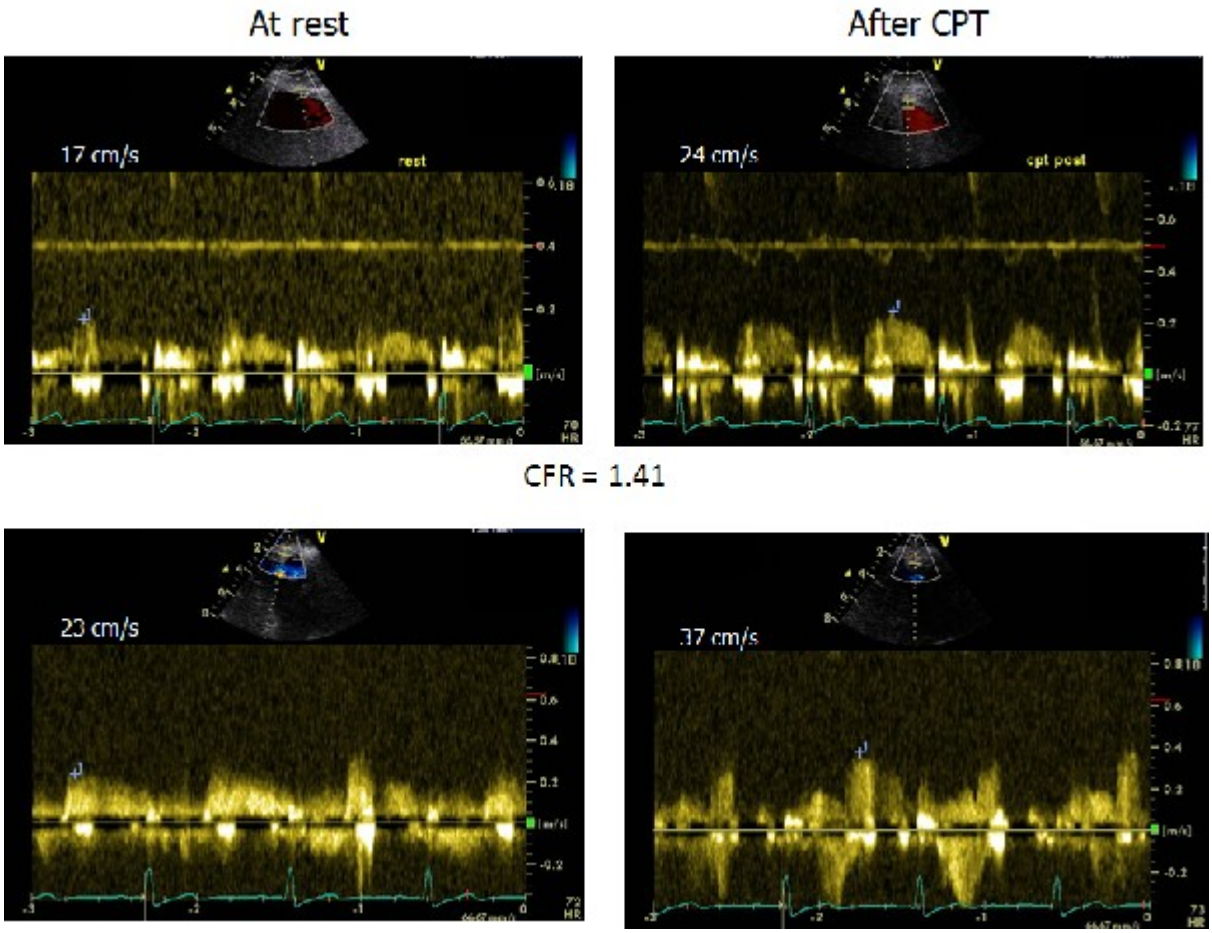

Fig.4 Cold pressure test (CPT)-induced coronary flow reserve at baseline and 24 h after the second recombinant human thyrotropin (rhTSH) injection in a single patient.

Upper panel: Coronary flow velocities at rest and after CPT at baseline.

Lower panel: Coronary flow velocity at rest and after CPT 24 h after the second rhTSH injection.

Ippolito S, Ippolito R, Peirce C, Esposito R, Arpaia D, Santoro C, Pontieri G, Cocozza S, Galderisi M, Biondi B. Recombinant human thyrotropin improves endothelial coronary flow reserve in thyroidectomized patients with differentiated thyroid cancer. Thyroid. 2016; 26(11):1528-1534 (64)

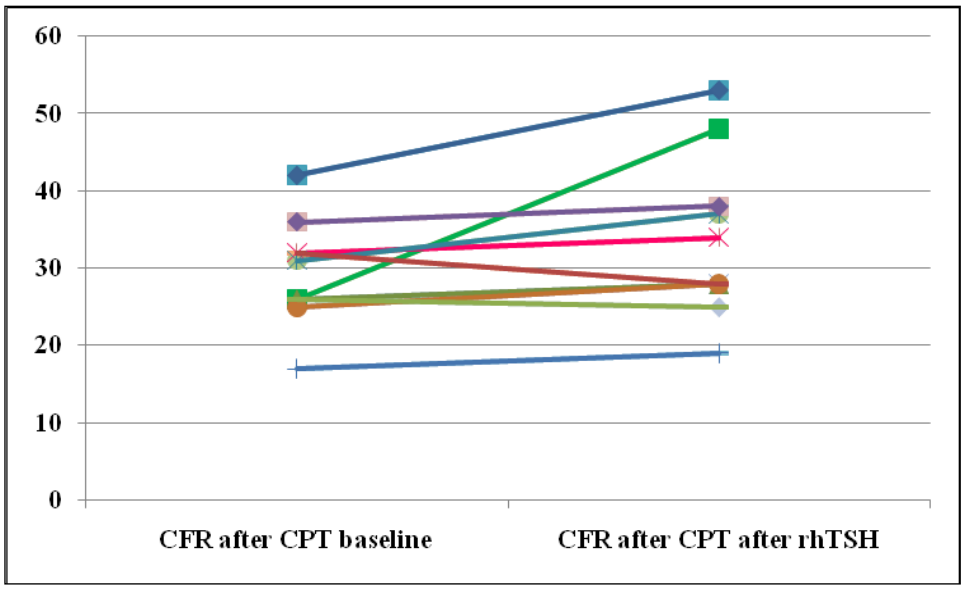

Fig.5 Changes in coronary flow after CPT at baseline and 24 h after second rhTSH administration.

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