The association between thyroid parameters and insulin resistance in patients with autoimmune thyroid disease

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UNIVERSITY OF SPLIT
SCHOOL OF MEDICINE

Paul Philipp Kastner

THE ASSOCIATION BETWEEN THYROID PARAMETERS AND INSULIN RESISTANCE IN PATIENTS WITH AUTOIMMUNE THYROID DISEASE

Diploma Thesis

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LIST OF ABBREVIATIONS

AIM2 – absent in melanoma 2

AITD – Autoimmune Thyroid Disease

APC – Antigen Presenting Cell

ASC - Apoptosis-associated speck-like protein containing a CARD; PYCARD

ATPase – Adenosine-tri-phosphatase

BACH2 - Broad complex-tramtrack-bric a brac and Cap'n'collar homology 2

CD – Cluster of differentiation

CTLA-4 – Cytotoxic T-lymphocyte-associated Protein 4

D1 – Peripheral deiodinase type 1

D2 – Peripheral deiodinase type 2

D3 – Peripheral deiodinase type 3

DM – Diabetes Mellitus

DUOX 1 – Dual oxidase 1

DUOX 2 – Dual oxidase 2

EPO – Erythropoietin

FLT3 – Fms like tyrosine kinase 3

GAGs – Glycosaminoglycans

GD – Graves' Disease

HLA-DR – Human leukocyte antigen-DR isotype

HT – Hashimoto's Thyroiditis

IFN-α – Interferon-alpha

IgG – Immunoglobulin G

IGF1-R – Insulin-like growth factor 1-Receptor

IL - Interleukin

IR – Insulin Resistance

LAG-3 – Lymphocyte activation gene 3

LT4 – Levo-Thyroxine

LT3 – Levo-Triiodothyronine

lncRNA – Long non-coding ribonucleic acid

MetS – Metabolic Syndrome

miRNA – Micro ribonucleic acid

NIS – Sodium – iodide - symporter

NADPH – Nicotinamide adenine dinucleotide phosphate

ncRNA – Non-coding ribonucleic acid

NLRC4 - NOD-like receptor family CARD-containing 4

NLRP – NOD-like receptor protein

PCOS – Polycystic ovary syndrome

PPT – Postpartum Thyreoditis

PTPN22 – Protein tyrosine phosphatase non-receptor type 22

ROC – Receiver operating characteristic

ROS – reactive oxygen species

SD – Standard deviation

SERCA - Sarcoplasmic/endoplasmic reticulum calcium ATPase

SNP – Single-nucleotide polymorphism

T3 - Triiodothyronine

T4 – Thyroxine

TBAbs – Thyroid blocking antibodies

TBG – thyroxine-binding globulin

Tc cells – Cytotoxic T cells

Tg - Thyroglobulin

TgAb – Thyroglobulin Antibodies

TH – Thyroid Hormones

Th cells – T-helper cells

TNF–α – Tumor necrosis factor-alpha

TPO – Thyroid peroxidase

TPOAb – Antibody against Thyroid peroxidase

TR – Thyroid hormone receptor

TRAK – Thyroglobulin receptor Antibody

TRE – Thyroid hormone response element

TRH – Thyroid releasing hormone

TSAbs – Thyroid stimulating antibodies

TSH – Thyroid stimulating hormone, Thyreotropin

TSHR – Thyrotropin receptor

TSI – Thyroid stimulating immunoglobulin

TTR – Transthyrexin

TyG Index – Triglyceride glucose index

UCP-1 – Uncoupling protein 1, Thermogenin

1. INTRODUCTION

Autoimmune thyroid diseases (AITD) belong to the most common disorders with autoimmune etiology in modern western societies (1–3). They clinically present as different entities, the two main ones known as Hashimoto's thyroiditis (HT) and Grave's disease (GD). They are characterized by disturbance of thyroid function and cause states of hypo – or hyperthyroidism, respectively (4). They ultimately lead to thyroid gland destruction due to altered immunologic action of various immune cells and the presence of autoantibodies (5–7). Based on genetic susceptibility, the onset of disease can be triggered by several factors and they most commonly affect women in the first half of life (8,9).

It is well known, that any disturbance of thyroid function will have direct or indirect effects on different metabolic processes throughout the body, including the homeostasis of insulin and glucose (10). Disturbance of insulin and glucose function might provoke the emergence of insulin resistance (IR), a pathologic state in which insulin can't exert its full effects on target cells, thereby inducing states of hyperglycemia and promoting different other pathophysiologic processes with severe consequences, like atherosclerosis (11,12). The main thyroid parameters to represent thyroid function are TSH, fT3, and fT4. Disturbances of equilibria are induced by any thyroid disease, including AITD, or by medications thyroid patients receive as (supplementation) therapy (13,14). As already mentioned, these alterations of thyroid hormone homeostasis might promote insulin resistance by a variable degree.

In the past, researchers tried to connect the risk of developing insulin resistance and the presence of autoimmune thyroid disease. There is not only evidence that the presence of thyroid autoantibodies can be connected with IR, metabolic syndrome, and obesity, but also that there are linear relationships between thyroid parameters and IR (15–20). Also, the causality of associations might be different, meaning thyroid dysfunction originating from alteration of nutritional status or body composition (21). So far, researchers' results worldwide have been partly incongruent, and the need for further evaluation of those associations exists.

In addition, several factors might influence the peripheral conversion of the prohormone T4 into the active hormone T3 by tissue deiodinases (22). Focusing on this essential contribution to overall thyroid function has long been neglected by medical research. However, new methods are available to quantify former processes and integrate them into scientific considerations.

This study aimed to evaluate further some of the relationships involving insulin resistance discovered by recent studies and to include focusing on possible deiodinase conversion disturbances in autoimmune thyroid disease.

1.1. The Thyroid Gland

The thyroid gland is an endocrine organ in humans, involved in a huge variety of metabolic, cardiovascular, and developmental functions. By synthesis and secretion of thyroid hormones into the bloodstream, it stimulates intracellular signaling cascades in target cells throughout many types of tissues in the whole body. Thyroid hormones are known as thyroxine (T4) and triiodothyronine (T3) (23).

1.1.1. Anatomy

Anatomically, the thyroid gland sits anterior to the trachea and is built up of a central isthmus that connects a right and a left lobe. Due to its' critical physiologic role, the thyroid gland is highly vascularized and receives its blood supply from branches of the external carotid artery, namely the superior and inferior thyroid arteries, and is drained by superior, middle, and inferior thyroid veins. Sympathetic fibers deliver vasomotor innervation (24).

Several units can be seen histologically in the thyroid tissues, which are described as thyroid follicles. The thyroid follicles are structures lined by thyroid follicular epithelial cells and consist of a lumen that acts as a scaffold, filled with colloid, containing big proteins called thyroglobulin. Another type of cells spread throughout the matrix surrounding the follicles. Parafollicular C Cells produce a peptide hormone, called calcitonin (a parathyroid hormone antagonist) (23).

1.1.2. Physiology

1.1.2.1. Thyroid Hormone Production

Hormones secreted by the thyroid gland are iodothyronines. These proteins are products of a complex cascade, involving the integration of the trace element iodine (I2) during different steps of synthesis across thyroid epithelial cells and the follicular lumen (25). For this reason, deficient or excessive iodine supply can lead to severe thyroid gland dysfunction (26). In people living in iodine-sufficient regions, the thyroid gland actively takes up 60-95μg of circulating iodide (I-) daily via a sodium-iodide symporter called NIS (26). Iodide is moved across the cell into the follicular lumen by pendrin, an iodide/chloride transporter. Thyroid peroxidase (TPO), an apical membrane enzyme complex, catalyzes the oxidation of iodide (I-) to iodine (I2) (the electron acceptor is H_2O_2, which is generated by dual oxidases, $DUOX_1$ and $DUOX_2$, in the follicular lumen), its incorporation into follicular thyroglobulin and eventually coupling of iodinated tyrosine molecules to form T4 and T3 (25). 80-90% of the formed products are 3,5,3′,5′-tetraiodothyronines, prohormones, known as thyroxine or T4. Approximately 10-20% of the output is 3,5,3′-triiodothyronine or T3, the active thyroid hormone (23,27). Additionally,

a small amount (less than 1%) of 3,3´,5´-triiodothyronine or "rT3", which is inactive, is secreted as well, (23).

1.1.2.2. Peripheral Deiodinases

Since the secretory output of the thyroid gland mainly consists of the prohormone T4, the supply of the active form of thyroid hormones (T3) and therefore the function of the thyroid axis depends largely on peripheral deiodinases. These thyronine-specific enzymes sit in target tissues to execute the local conversion of T4 to the active form T3 by the process of deiodination (23,27).

Three types of peripheral thyronine deiodinases are known. Type 1 (D1) is expressed in highly perfused organs like the liver and kidneys and is critical for the regulation of thyroid hormone function (28). D1 supplies T3 for all organs, which by themselves have low or absent T3 generation capabilities, via the bloodstream (23). Type 2 (D2) located in the CNS, and expressed by glial cells, provides steady concentrations of T3 in the brain and spinal cord. It is also found in pituitary thyrotropes where it modulates TSH secretion, thereby acting as a mediator of feedback control of the thyroid hormonal axis (29). Furthermore, a third type of deiodinases can be found. D3 converts T4 into the inactive reverse T3 or "rT3" and helps to maintain adequate hormone levels (23).

1.1.2.3. Bloodstream Transport

In the bloodstream, secreted T3 and T4 mainly bind to circulating proteins, to establish a steady reservoir, which can balance the variability of thyroid gland function and prevent unwanted loss of iodine via the urinary system (30). The bigger part of TH (70%) is bound to thyroxine-binding globulin (TBG), while a smaller portion (10-15%) is bound to a protein responsible for transportation into the CNS, transthyretin (TTR) (23). Albumin and lipoproteins bind the remaining portion (10-15% and 3%, respectively). Only about 0.03% of T4 and 0.3% of T3 can be found free in plasma (23).

1.1.2.4. Feedback Control

Besides their effects on target tissues, free circulating TH are involved in controlling thyroid functioning by a modulatory feedback mechanism. Free T4 (via D2) and free T3 have an inhibitory effect on TRH releasing CNS (parvocellular) neurons and on pituitary thyrotropes (23). Consequently, less TSH is synthesized and released into the bloodstream in the presence of high plasma T3 and T4 levels and vice versa (31). This relationship is log-linear, meaning only minor changes in circulating T3 or T4 can result in major TSH alterations (31). TSH impacts thyroid functions in various ways, including pinocytosis of colloid droplets, secretion

of TH, iodide uptake, TPO activity increase, NADPH supply, gene expression of NIS, Tg, and TPO, and hypertrophy and hyperplasia of follicular cells (23).

1.1.2.5. Effects on Target Tissues

TH act on almost all tissues in the human body. They act directly or indirectly on a wide variety of cells, but also modulate the function of other hormones or neurotransmitters (23). Mechanism of action in target cells can be genomic, meaning influencing gene transcription mainly by binding to thyroid hormone nuclear receptors (TRs) that subsequently interact with specific gene sequences called thyroid hormone response elements (TREs). Pathways activated by TH binding to cell-membrane, cytoplasmic, or mitochondrial binding sites represent another well-known concept of TH action and are known as the non-genomic or extra-nuclear pathways. Ultimately, these mechanisms lead to stimulation or repression of gene transcription and therefore protein synthesis, and to modification of intracellular signaling cascades (32).

The most studied effects of TH are those on the cardiovascular system, the basal metabolic rate, thermogenesis, the respiratory system, skeletal muscle, (autonomic) nervous system, growth and maturation, bones, skin, and endocrine or reproductive organs (25).

On the heart, TH have positive chronotropic, inotropic, and lusitropic effects. Those effects are mediated by increased cellular receptivity to catecholamines and enhancement of intracellular calcium metabolism. The decrease in total peripheral resistance by dilating arterioles contributes to the increase in pulse pressure. In addition, TH stimulates the renin-angiotensin-aldosterone axis, thereby promoting fluid and sodium retention and increasing total blood volume (23).

Thyroid function also strongly affects metabolic processes throughout the body. By the genomic effect of stimulating Na+/K+ ATPase expression, basal metabolic rate and heat production get augmented, concurrently rising oxygen and energy consumption (25). In addition, T3 is an important augmenter in providing substrates for oxidation processes. The uptake, synthesis, and turnover of glucose and free fatty acids get stimulated. To a lesser extent, the same effect applies to protein metabolism. Lipolysis, proteolysis, and gluconeogenesis profit from a synergistic stimulatory effect of thyroid hormones and catecholamines. Low-density lipoprotein cholesterol levels decrease, due to an augmented production of bile acids and secretion of the latter (23).

Uncoupling protein-1 (UCP1), also known as Thermogenin, is responsible for physiologic heat production in brown fat tissue. Its' function gets optimized by T3 and D2 (10). In addition, TH have a stimulatory effect on membrane pumps like Na+/K+ ATPase and SERCA, thereby promoting heat generation even further (23).

Several other essential clinical effects of TH have been described: Increase in respiratory rate, minute ventilation, ventilatory response, EPO production, promoting growth and maturation, neurologic development, proper bone formation, tooth development, enhancement of cognitive functions, motility of the gastrointestinal tract, regulating ovarian cycle, spermatogenesis, maintaining pregnancy and maintaining the availability of steroid hormones (23,25,33,34).

1.2. Autoimmune Thyroid Diseases (AITD)

The knowledge about the connection between autoimmunity and disease of the thyroid gland is well established in modern medicine. It had its origins at the beginning of the 19th and 20th centuries when the phenotype and symptoms of these diseases were depicted by physicians for the first time. H. Hashimoto 1912 first described his observations of hypothyroid patients (35). Even earlier, characteristics of Grave's disease were delineated by R.J. Grave in 1835 (36). Since then, autoimmune genesis has been attributed to the two entities and extensive research has been accumulated to continuously extend our knowledge of both forms of thyroid disease. Clinically, AITD presents mainly either as Hashimoto's thyroiditis (HT) or Graves' disease (GD) (4). In addition, there are some less prevalent forms of AITD, including postpartum thyroiditis, drug-induced thyroiditis, or polyglandular autoimmune syndromes (37). In the following, AITD refers to the two main entities and the two main objectives of this research, HT and GD.

1.2.1. Epidemiology

The importance of autoimmune diseases is overly present in our modern world since they affect 7-10% of the global population (1,38,39), and prevalence and incidence are rising steadily in western societies (1). Although there is no clear evidence if both main entities of autoimmune thyroid disease, namely Hashimoto's thyroiditis and Grave's disease, tend to follow this general trend (2), taken together, they represent the most common autoimmune diseases worldwide (3,9,38). Striking evidence is found on a huge female sex dominance for both entities, approaching ten times the incidence of the diseases in males, which can, among others, be linked to relevant differences in adipokine levels (3,9). Studies performed in the past decades also show a strong correlation between incidence and geographic distribution, linked to the supply of micronutrients, like iodine or selenium (2,40,41), and ethnical differences (2,3).

1.2.2. Genetics

Autoimmune thyroid disease is multifactorial in its' pathogenesis. Studies suggest a predisposing genetic influence of 79% and an influence by environmental triggers of 20% (8).

One group of susceptibility genes lies in the HLA-DR gene locus. Similar to mechanisms of genetic changes in the pathogenesis of Diabetes Mellitus Type 1, a specific pocket HLA-DR amino acid signature is responsible for altered peptide binding and subsequently altered involvement and initiation of antigen-presenting cells in AITD (5,8,42).

Immune-regulatory genes could also play a central role in predisposing to AITD. A very well-proven finding is the association of CTLA-4 polymorphisms and autoimmunity. CTLA-4 polymorphisms seem to be directly involved in the pathogenesis of GD and HT by influencing thyroid antibody production, modifying T-cell responses, and affecting neighboring genes (43–49). Another strong correlation was found between a CC genotype SNP (single nucleotide polymorphism) of the CD40 gene and the risk of developing GD with high thyroid antibody levels (5,8).

In addition, numerous studies showed increased susceptibility to AITD through genetic morphologic variants of the disease-specific TSH-receptor genes (TSHR), thyroglobulin genes (Tg), other immune-modifying genes including tyrosine phosphatase encoding genes (PTPN22), tyrosine kinase encoding genes (FLT3), interleukin-2-receptor-α genes (CD25/IL-2Rα), transcription factor encoding genes (FOXP3), and genes involved in the process of TPOAb synthesis (TPO, BACH2) (5,7,8,49–52).

The precise mechanisms of how SNPs in the genes mentioned above ultimately lead to AITD are mostly still unknown and an active field of research. Besides these identified specific genes, new and ongoing studies point towards susceptibility patterns originating from changed expression patterns of ncRNAs like miRNAs and lncRNAs, which regulate dendritic cells, various T-cells, and B-cell populations (50).

Furthermore, epigenetic mechanisms like DNA methylation and histone modifications could play a substantial role in our understanding of the modulation and integration of genetic and environmental factors (53,54).

1.2.3. Environmental Factors

Infections have been identified to play key roles in triggering AITD (8,42,55). An extensively studied concept of inducing autoimmunity in different disease entities is molecular mimicry. In the past several pathogens have been identified that can trigger a human immune response targeted against self-antigens due to immunogenetic overlap of pathogenic epitopes and similarities in various human protein antigens (50). Another proposed concept is the bystander activation theory. In this case, local inflammatory mediators are released as a result of infection by a pathogen. These mediators ultimately induce T-cell reactivity against self-antigens (5,8).

An analysis of relevant studies identified the most important pathogens involved in these processes, potentially leading to the development of AITD. They include Borrelia, Yersinia, C. botulinum, R. prowazekii, H. pylori, Toxoplasma gondii, Bifidobacteria, Lactobacilli, C. Albicans, T. pallidum, and especially Hepatitis C virus (56).

Also relatable to initiation or progression of AITD is the inadequate supply of micronutrients like iodine, selenium, and vitamin D, as well as other external factors like alcohol consumption, smoking, stress & adverse life events, drug therapy with IFN-α, pregnancy or radiation exposure (8,8,50,57).

The composition of the human microbiome also moved into the focus of current research and seemingly influences the pathogenesis of AITD (50).

1.2.4. Pathogenesis

The postulated mechanism of developing AITD is losing immunological tolerance towards proteins expressed in thyroid tissue as a result of a complex interaction between genetic predisposition and the influence of environmental factors (50). In this scenario, CD4+ cells evade mechanisms of central and peripheral tolerance (failure of positive and negative selection in the thymus and failure of regulation and inhibition of autoreactive T-cells, respectively) and as a result, execute destructive immune reactions against thyroid immunogenic proteins (50).

As a consequence APCs present autoantigens, which lead to the infiltration of thyroid tissue by B-cells, cytotoxic T-cells, and CD4+ cells. CD4+ cells differentiate into pro-inflammatory Th1, Th2, and Th17 cells and into attenuated Treg cells, which fail to counteract the pro-inflammatory activities (50). In the recent past, it could be shown that certain subtypes of regulatory T-cells, namely T regulatory type 1 cells (CD4+, CD49+, LAG-3+, IL-10+), are diminished in number and function in patients with autoimmune thyroid disease (58).

In summary this cascade results in lymphocytic infiltration of the thyroid gland, the production of different antibodies against thyroid antigens, the release of different pro-inflammatory cytokines, apoptosis of thyrocytes, and eventually thyroid gland dysfunction (50). The importance of proinflammatory cytokines in the pathogenesis of autoimmune thyroid disease has been underlined by many studies in recent global research. A wide variety of these cytokines can be demonstrated in induction as well as effector phases of the immunologic reactions and also have specific different patterns of engagement in HT and GD (59).

Autoantigens mainly involved in the pathophysiologic processes of AITD development are Thyrotropin receptor (TSHR), Thyroid peroxidase (TPO), and Thyroglobulin (Tg) (50). The role of other antigens like sodium-iodide symporter (NIS) or pendrin remains under discussion

(50). Thyroid peroxidase antibodies have long been considered the best diagnostic indicator for autoimmune thyroid disease and can be detected in 90-95% of AITD patients (60). They usually are immunoglobulin G subclasses (IgG) with different associations with thyroid function. IgG2 and IgG4 dominance is believed to be a risk factor for apparent hypothyroidism in HT patients (61). It is under extensive debate to what specific extent these autoantibodies can be linked to the pathophysiologic processes in autoimmune thyroid disease. For example in HT, discordance between the presence of TPO-Abs and histopathologic findings has been shown recently (62). Furthermore, some researchers question which autoantibody has the highest prognostic or diagnostic value for autoimmune thyroid diseases; e.g. a recent study from Japan suggests, rather using TgAb in the diagnosis of HT than TPO-Ab, as has been widely accepted to be the standard (63).

In addition, the correlation of symptoms burden with the extent of autoimmune processes is not always completely obvious, as was shown by a meta-analysis of studies involving HT patients that couldn't find a definitive correlation between the quality of life and antibody levels (64). However, one study could show an increase in general health score and reduction of fatigue and chronic fatigue in patients with suppressed autoimmune processes (6).

1.2.5. Differences between Hashimoto's Thyreoditis and Graves' disease

HT presents with thyroiditis, destruction of follicular cells, presence of TPOAbs, TgAbs, and hypothyroidism. Th and Tc cells seem to mediate gland destruction by infiltration and cytokine release, which further amplifies the decline in thyroidal cells through mechanisms of proinflammatory cascades, resulting in pyroptosis (6,65). As global knowledge about these immune processes extends, focus on HT was recently drawn towards the role of IL-21, IL-18, IL-ß1, and inflammasome components NLRP1, NLRP3, NLRC4, AIM2, ASC, and caspase-1 (6).

Clinically, early in the progress of HT a state of hyperthyroidism has been reported many times, which is regarded as a result of the release of thyroid hormones by the acute inflammation and tissue destruction process (65). This state of hyperthyroidism is followed by the main and final manifestation of HT, hypothyroidism (65). Compared to Graves' disease, Hashimoto patients experience weight gain due to a diminished basal metabolic rate. Cold intolerance, dry skin, reduced sweating, and ptosis are results of impaired thermogenesis and autonomic nervous functions, as is applicable for bradycardia (23,66). General cerebral perfusion and cerebral glucose metabolism are reduced in HT. As a result, psychic symptoms like depressed mood, lethargy, sleepiness, and slowing of cognitive functions can be observed (65). Typically, HT patients also suffer from hair loss, skin irritations, constipation, menstrual

anomalies, and hematologic disturbances like anemia. In addition to that, there can be accumulation of mucopolysaccharides in connective tissues which draw water and sodium into the extracellular matrix due to their negative charge. The emerging so-called nonpitting myxedema is known for causing hoarseness, puffy features, pleural/pericardial or peritoneal effusions, joint stiffness, and cranial nerve entrapment (23,65).

GD presents with thyroiditis, T and B cell infiltration (less than in HT), hyperthyroidism, presence of stimulating TSHR antibodies (TSIs), and thyroid hyperplasia by follicular cell hypertrophy (7). Traditionally, Th2 cells were considered the main activators of GD autoimmunity, but recent observations suggest a common Th1-cytokine/chemokine axis for initiation of both forms of AITD (50). APC presentation of TSHR peptides triggers the activation of T and B cells which leads to plasma cell infiltration, cytokine release (especially IL-2 and IL-17), TSAb (TSI stimulatory subtype) production, and increased TH secretion (50). In addition to TSHR stimulating autoantibodies, TSHR blocking autoantibodies can be found in GD (TBAb) as well, modulating the degree of TSHR activation and explaining changing patterns of thyroid overactivity to underactivity also known as Graves' Alternans (67). In GD various amounts of TgAbs can be found and to 75% TPOAb, as well (50).

The clinical presentation of grave's disease is well described. Generally, it opposes HT in its phenotype by displaying weight loss, sleeplessness, heat intolerance, sweating, gastrointestinal malfunction, and psychic disturbances like anxiety and cognitive dysfunction (7,68,69). Swelling and hyperplasia of the thyroid gland can become visible in the anterior neck region and is known as goitre (23).

Still, symptoms can be very different among patients and frequently appear as mixed clinical pictures, since they strongly depend on the onset time, disease characteristics (silent, subclinical, overt disease forms), the overall duration of the dysfunction, and other unknown factors. Presentation of symptoms also varies with age, with cardiovascular symptoms dominating in people over the age of 50 (7,69). Effects on the cardiovascular system are mainly considered to be a result of increased sensitivity to catecholamines, mediated by intracellular actions of TH, increasing translocation of beta-receptors to the cell membrane. As a result, patients regularly present with palpitations, tachycardia, systolic hypertension, increased pulse pressure, and increased stroke volume. In the long term, untreated cases of GD, atrial fibrillation, and even heart failure can develop (70).

Since TSHR is expressed in extrathyroidal tissue (adipocytes, fibroblasts, extra orbital muscle), GD often includes Graves' ophthalmopathy, Graves' dermopathy (pretibial myxedema), thyroid acropachy, and Graves' thymus hyperplasia (7,50).

In Graves' ophthalmopathy, activation of adipocytes and fibroblasts is thought to be mediated by autoantibodies directed against TSHR and IGF1-R in orbital tissues. Subsequently, the production of GAGs and hyaluronic acid synthesis increases. These osmotic changes together with proliferative changes and inflammation ultimately lead to swelling of retrobulbar tissues. Temporary swelling mediated by congestion and periorbital edema may be reversible but could if left untreated, lead to irreversible fibrotic changes. Patients might complain about loss of sight, diplopia, eyelid retraction, keratitis, or proptosis. The tissue expanding alterations get visible over time, leading to eye protrusion out of the orbita, commonly known as exophthalmos (7,71,72).

Transformation of HT to GD or vice versa is a known phenomenon that is topic of current research. Information on the incidence of patients experiencing a conversion of the two entities is sparse. Transformation of HT to GD is described as rarely occuring, as opposed to the more frequent transformation from GD to HT. Latest scientific papers propose a common pathogenic role of regulatory T cells, being able to alter immunologic processes towards the one or other side of the autoimmune disease spectrum (73–77).

1.2.6. Other Forms of Thyroid Autoimmunity

As already mentioned, besides the well-known main entities of AITD, other less common forms exist. Postpartum thyroiditis (PPT) affects up to 5-9% of pregnant women and is favoured by the Th2 to Th1 immune response shift occuring in the postpartum time (37). The disease manifests within this period and occurs mostly transiently, attenuating within 12-16 months (78). PPT presents with positive TPOAbs, features signs of lymphocytic gland destruction, and clinically often shows up as hyperthyroidism (in 32%), hypothyroidism (in 43%), or initial hyperthyroidism followed by hypothyroidism (25%) (78). A high risk of developing PPT is associated with positive TPOAbs in the first trimester, the coexistence of Diabetes Mellitus Type 1, and having a prior history of PPT (78).

1.2.7. Coexisting Autoimmune Diseases

There is a high likelihood for AITD to co-exist with other autoimmune disturbances in the same patient (79,80). Diabetes Mellitus Type 1, celiac disease, and rheumatoid arthritis are the most common representatives (80). Percentages of co-existence range up to 18,5% in GD and 27,8% in HT, which is why other clinical symptoms, not specifically typical for AITD, appear regularly in these patients (80).

1.3. The Link between Metabolic Status, Thyroid Dysfunction, and Autoimmunity

It is well known that thyroid function plays a central modulatory role in regulating metabolic processes involved in energy expenditure, and lipid or glucose homeostasis (25,31). Thus, dysfunction of the thyroid gland has been associated with obesity, cardiovascular, and common endocrinologic diseases including metabolic syndrome (MetS) and diabetes mellitus (DM) by researchers in the past (81–87).

A well-established concept is the correlation between hypothyroidism and an increase in serum triglycerides and LDL production by the liver. A recent German study supported the evidence that also autoimmune thyroid disease as a single factor can be associated with liver dysfunction, NAFLD development, and dyslipidemia. Based on the results, it could be proven that Hashimoto's disease acts as aggravating factor, while Graves' disease acts as a protective factor on this disturbance of liver function (88).

Furthermore, reduced HDL serum levels, elevated blood pressure, and increased abdominal obesity have been linked to TPO antibody levels. In a conclusion, even in euthyroid subjects, thyroid autoimmunity has been identified as a major risk factor for metabolic syndrome (81).

Interestingly, thyroid dysfunction and obesity seem to have a bidirectional influence, potentially impacting the emergence and the progress of each other (17). While there has been strong evidence for obesity being a risk factor for hypothyroidism on its own (89), recent studies indicate an association between obesity and thyroid autoimmunity, as well, since higher TPOAbs are found in obese patients, which could be related to high serum levels of IL-6, TNF-alpha, and leptin in obese patients (21). Obesity increases serum levels of adipokines, which can propagate the emergence of AITD (21). In addition, it has been shown that there is a correlation between TPOAbs and obesity, but not between TgAbs and obesity (89).

Strongly connected to obesity is the dysfunction of glucose and insulin homeostasis and the prevalence of concomitant insulin resistance, which is described in the next chapter. Indirect effects of thyroid hormones on insulin and glucose include weight changes, altered body fat composition, and appetite regulation. Direct effects are commonly regarded as acting against insulin on the hepatic level (by stimulating hepatic gluconeogenesis and glycogenolysis) and synergistically with insulin on muscle and adipose tissue, e.g., by stimulating GLUT-4 translocation (25,90).

1.4. Insulin Resistance (IR)

Disturbances in glucose and insulin homeostasis are one of the biggest issues of modern medicine since pathologic alterations of these balanced mechanisms delineate major risk factors

for developing severe chronic conditions like diabetes mellitus and cardiovascular disease, especially due to the associated atherogenic potential (11,12,70).

Insulin, known since 1921, is crucial in lowering serum glucose levels and mediating anabolic processes throughout the body directly and indirectly. The main target tissues to execute its effects on are skeletal muscle, white adipose cells, and hepatocytes (91). Dysfunction of insulin effector pathways leads to severe metabolic alterations like hyperglycemia or dyslipidemia. Those imbalances can be introduced by the development of insulin resistance, meaning the inability of insulin to exert its function on target cells (91). Until today, the mechanism of the emergence of insulin resistance is not fully understood. Proposed mechanisms are among others defects in intracellular signaling pathways, activation of nutrient-sensitive pathways, ROS involvement, proinflammatory factors, lipid accumulation, or engagement of regulatory glucogenic feedback systems (11,12,91,92). Insulin resistance is present in patients in a prediabetic state and coexists with different aspects of the metabolic syndrome, like obesity. A high amount of abdominal fat tissue, a sedentary lifestyle in combination with overnutrition (excess glucose and fructose loads), and bad eating habits (eating at night, eating fast, eating frequent small meals) are currently being regarded as major risk factors for insulin resistance and associated progression of metabolic disturbances into metabolic syndrome (93–99).

Many other influences could additionally disturb insulin metabolism and propagate the development of insulin resistance. Widely accepted risk factors today are positive family history of diabetes, ethnical background, age, hormones, steroid use, some drug therapies (ß-blocker, diuretics, anti-diabetics, anti-psychotics, anti-depressants & anti-convulsants, statins, oral contraceptives, chemotherapeutic agents, HIV therapy), poor sleeping habits, smoking, hepatitis C infection (100–103).

The connection of thyroid dysfunction or autoimmune thyroid disease with the emergence of insulin resistance is under active scientific discussion. Conventionally, it was shown that hyperthyroidism induces central (hepatic) insulin resistance, going hand in hand with previously described stimulatory effects of TH on hepatic glucose output. Much harder to explain were findings of past studies of insulin resistance in states of hypothyroidism, which are now thought to be mediated by peripheral (muscle) insulin resistance (90,104). Regarding thyroid function parameters, a associations of TSH, fT3, and fT4 levels with HOMA-IR and cholesterol levels have been found previously (82,105).

Interestingly, it has also been shown that thyroid autoimmunity independently of thyroid function is associated with increased insulin resistance (83). In addition, other studies have

shown a significant association between an autoimmune hypothyroid state and insulin resistance (20). While an exact explanation of these relationships remains unknown, it has been proposed that increased proinflammatory cytokine levels in insulin-resistant patients, could trigger the production of TPOAbs (20).

The available literature is often contradictory and despite reasonable medical research efforts, our understanding of these processes remains poor until today. Continuously more scientific work is currently being performed to shed more light on these interrelationships, which will also be the objective of our research.

2. OBJECTIVES

Aims of the Study

1. Our main goal was to observe relationships between laboratory thyroid parameters fT3, fT4, TSH, TPOAb, TgAb, SPINA-GT, SPINA-GD, TTSI, TSHI, and the homeostatic model assessment of insulin resistance, HOMA-IR. We were particularly interested in comparing these associations in patients with and without autoimmune thyroid disease.
2. Our secondary goal was to examine possible influences connected to fT3 serum level in our patient collective, especially due to our suspicion of fT3 serum level alteration in autoimmune thyroid patients.

Hypotheses

We formulated two main hypotheses for our research.

1. Thyroid parameters are associated with insulin resistance in autoimmune vs. non-autoimmune thyroid patients
2. Thyroid autoimmune disease is associated with lowered fT3 serum levels

3. SUBJECTS & METHODS

3.1. Data Source and Study Objects

In this retrospective study, a sample was taken from a databank of previously anonymized patients who have been in therapy at the endocrinology department of the main regional hospital of upper Franconia/southern Thuringia, Klinikum Coburg, spanning a time frame from the beginning of 2015 until the end of 2021. The databank was created from information collected on individual appointment dates and from patients' electronic medical records.

On these appointment dates, each patient received a self-anamnesis form, an ultrasound examination, a blood drawing, and measurements of blood pressure, heart rate, weight, and height. Patients have been advised to keep an overnight fasting state before the venous blood drawing to ensure accurate blood sampling results.

The study has been permitted by the bavarian ethics committee (Bayrische Landesärztekammer), the ethics committee of the University of Split, and the internal review board of the MedicalSchool, REGIOMED.

3.2. Inclusion and Exclusion Criteria

For our study, we included all patients with existing HOMA-IR blood analyses and thyroid function tests (TSH, fT3, fT4), independently of disease status. We included euthyroid subjects as well as patients being in a state of hypothyroidism or hyperthyroidism, being under current treatment of thyroid hormones or thyreostatics, as well as patients who have undergone thyroidectomy or thyroid ablation therapy. We didn't exclude patients having comorbidities.

We excluded currently pregnant patients and patients with thyroid malignancy. We also excluded patients missing the required laboratory data (Insulin, glucose, HOMA-IR, TSH, fT3, fT4).

3.3. Measurements

Clinical measurements (blood pressure, pulse, height, weight, blood samples) has been taken from trained co-workers in our endocrinology department. On their appointment, patients have been measured and weighted with a ChiroMed/Seca device (Seca GmbH, Hamburg, Germany), which is a balanced scale with fixed meter on the wall. Height has been measured in cm with 0.5 step accuracy. Weight has been measured (with patients wearing light clothes) in kg with 0.1 step accuracy. These data have been used to calculate the BMI of each patient with the formula "$BMI = \frac{weight\ (kg)}{height\ (m)^2}$ ". Blood pressure and pulse have been obtained from our patients by the same trained nurse following current standards of blood pressure measuring (Pat. seated for five minutes before measurement; pat. is not allowed talking during measurement; using

measuring calf of correct size and correct position) with a Boso Medicus Uno D-72417 device (BOSCH + SOHN GmbH u. Co. KG, Jungingen, Germany).

Laboratory analyses of thyroid parameters have been performed by the hospital laboratory in Coburg, while analyses of thyroid antibodies, insulin, and glucose have been performed by external laboratories. There was a change in the external laboratory at the beginning of May 2018, involving a change of devices used for the analysis of insulin and glucose, but not for thyroid autoantibodies (before May 2018: MVZ Labor PD Dr. Volkmann und Kollegen, Karlsruhe, after May 2018: MedLab, Bamberg).

TSH, fT3, and fT4 serum levels have been determined in the hospital laboratory by using an electrochemiluminescence immunoassay (ECLIA) on a Cobas e601 system (ROCHE Diagnostics GmbH, Mannheim, Germany). Reference ranges used: TSH: 0.270-4.20 µIU/ml, fT3: 2.0-4.4 pg/ml, fT4: 0.93-1.7 ng/dl.

Measurements of thyroid autoantibodies were conducted by MVZ Labor PD Dr. Volkmann und Kollegen, Karlsruhe, and MedLab, Bamberg, using TRACE technology on the ThermoFisher Kryptor compact PLUS (B·R·A·H·M·S GmbH, Thermo Fisher Scientific, Hennigsdorf, Germany). Reference ranges used by MVZ Labor PD Dr. Volkmann und Kollegen, Karlsruhe: TPOAb: < 9 IU/ml, TgAb: < 4 IU/ml, TSAb: < 1.5 IU/L. Reference ranges used by MedLab, Bamberg: TPOAb: 1-16 IU/mL, TgAb: 5-100 IU/ml, TSAb: < 1.8 IU/L.

Before May 2018 (MVZ Labor PD Dr. Volkmann und Kollegen, Karlsruhe), glucose levels were detected by hexokinase method, using a Cobas c501 system (ROCHE Diagnostics GmbH, Mannheim, Germany). Reference ranges used: 74-106 mg/dL. Insulin was analyzed by immunoassay on an Immulite 2000 device (Siemens Healthcare GmbH, Erlangen, Germany). Reference ranges used: 2.0-25.0 mU/L.

After May 2018 (MedLab, Bamberg), glucose was analyzed by hexokinase method on a Cobas 8000 (ROCHE Diagnostics GmbH, Mannheim, Germany). Insulin was analyzed by electrochemiluminescence immunoassay on a Cobas e801 (ROCHE Diagnostics GmbH, Mannheim, Germany). Reference ranges used: 2.6-25.0 mU/L.

Comparability between the glucose measurements was given. Comparability of insulin measurements was not given initially, but the laboratory provided a correction factor (based on internally performed regression reference analyses) to ensure comparability achieved by transformation. The correction factor provided here was: Cobas_INSU = 1.15 * Immulite_INSU + 0.988.

3.4. Definitions

The status of autoimmune thyroid disease has been established exclusively by definition of the presence of thyroid autoantibodies against thyroid peroxidase (TPOAb > 16 IU/ml). For this reason, thyroid autoimmunity and TPOAb positivity will be used synonymously throughout the remaining text.

We used the following reference ranges for classifying our study population into different thyroid function groups:

- Euthyroidism: TSH: 0.27-4.2 µIU/ml, fT3: 2.0-4.4 pg/mL (3.1-6.8 pmol/L), fT4: 0.93-1.7 ng/dl (12.0-22.0 pmol/l)
- Hypothyroidism: TSH > 4.2 µIU/ml, fT3 < 2.0 pg/ml or fT4 < 0.93 ng/dl.
- Hyperthyroidism: TSH < 0.27 µIU/ml, fT3 > 4.4 pg/ml or fT4 > 1.7 ng/dl.
- Subclinical hypothyroidism: TSH > 4.2 µIU/ml concomitant with euthyroid fT3 and fT4 values.
- Subclinical hyperthyroidism: TSH < 0.27 µIU/ml concomitant with euthyroid fT3 and fT4 values.

Nutritional status was classified after the NIH/WHO obesity classification according to BMI (106):

- < 18.5 kg/m2: Underweight
- 18.5-24.9 kg/m2: Normal weight
- 25.0-29.9 kg/m2: Pre-obesity
- 30.0-34.9 kg/m2: Obesity class 1
- 35.0-39.9 kg/m2: Obesity class 2
- > 40 kg/m2: Obesity class 3

3.5. Usage of SPINA-Thyr to Analyze Thyroid Function

The importance of the activity of deiodinases in thyroid disease has been pointed out clearly in the text before. In recent years, a leading study group from Germany, introduced mathematical methods to calculate new function parameters from serum levels of fT3, fT4, and TSH. Designed for research purposes, SPINA-Thyr is a calculator of thyroid hormone production capacity (SPINA-GT) and peripheral deiodinase activity (SPINA-GD). Moreover, it allows the determination of the TSH Index (TSHI) and the Thyrotroph thyroid hormone resistance index (TTSI) (107–109). The formulas used have been well established and described in several studies (22,110–113).

Quantification and measurements of these processes could be of great importance in improving the understanding of pathologic processes in patients with any kind of thyroid disease. We were particularly interested in measuring and comparing peripheral deiodinase activity since in the practice, a tendency of low fT3 values despite adequate supplementation seemed to be observable in patients with AITD. Following the concept proposed by Hoerman *et al.*, we allocated patients also to being poor, intermediate, or good converters (14).

3.6. Usage of HOMA-IR to Estimate Insulin Resistance

Insulin resistance describes a diminished possible physiologic effect of insulin on target cell membranes, e.g., on hepatic cells mediating the inhibition of gluconeogenesis or on muscle cells mediating glucose uptake from the bloodstream. This concept has been widely accepted and used by researchers and clinicians likewise and was first postulated in 1936 (103).

Since then, efforts have been undertaken on finding a method to measure and display this possible pathogenic alteration of insulin function. The glucose clamp technique has been successfully used since 1979 and represents the gold standard to assess insulin resistance and ß-cell sensitivity to glucose and insulin (114). Nevertheless, due to inconvenience and uneconomical characteristics of the prior mentioned method, the most practicable technique used today is the HOMA-IR formula, first published in 1985, which estimates insulin resistance by a calculation based on plasma insulin and plasma glucose levels (115).

In this study, HOMA-IR was calculated (HOMA-IR = fasting insulin (mU/L) * fasting glucose (mg/ dL)/405) to estimate the prevalence of IR in autoimmune thyroid patients and possible interactions of thyroid hormone levels with the degree of severity of IR.

3.7. Groups for Statistical Analyses

To preserve the most significant possible sample size, we decided to build two main groups for the two main different analyses referring to our hypotheses.

For analysis of thyroid parameters, we excluded all patients with LT3 substitution, Iodine supplementation, thyreostatic therapy, prior thyroid surgery, or patients with missing information (Group 1).

For analysis related to insulin resistance, we excluded patients with polycystic ovarian syndrome, Diabetes Mellitus type 1 or type 2, patients with any other endocrinologic comorbidity, and patients under the influence of drug therapy known for promoting insulin resistance (anti-diabetics, statins, corticosteroids, ß-blockers, thiazide diuretics) (Group 2). A visual representation is given in Figure 1.

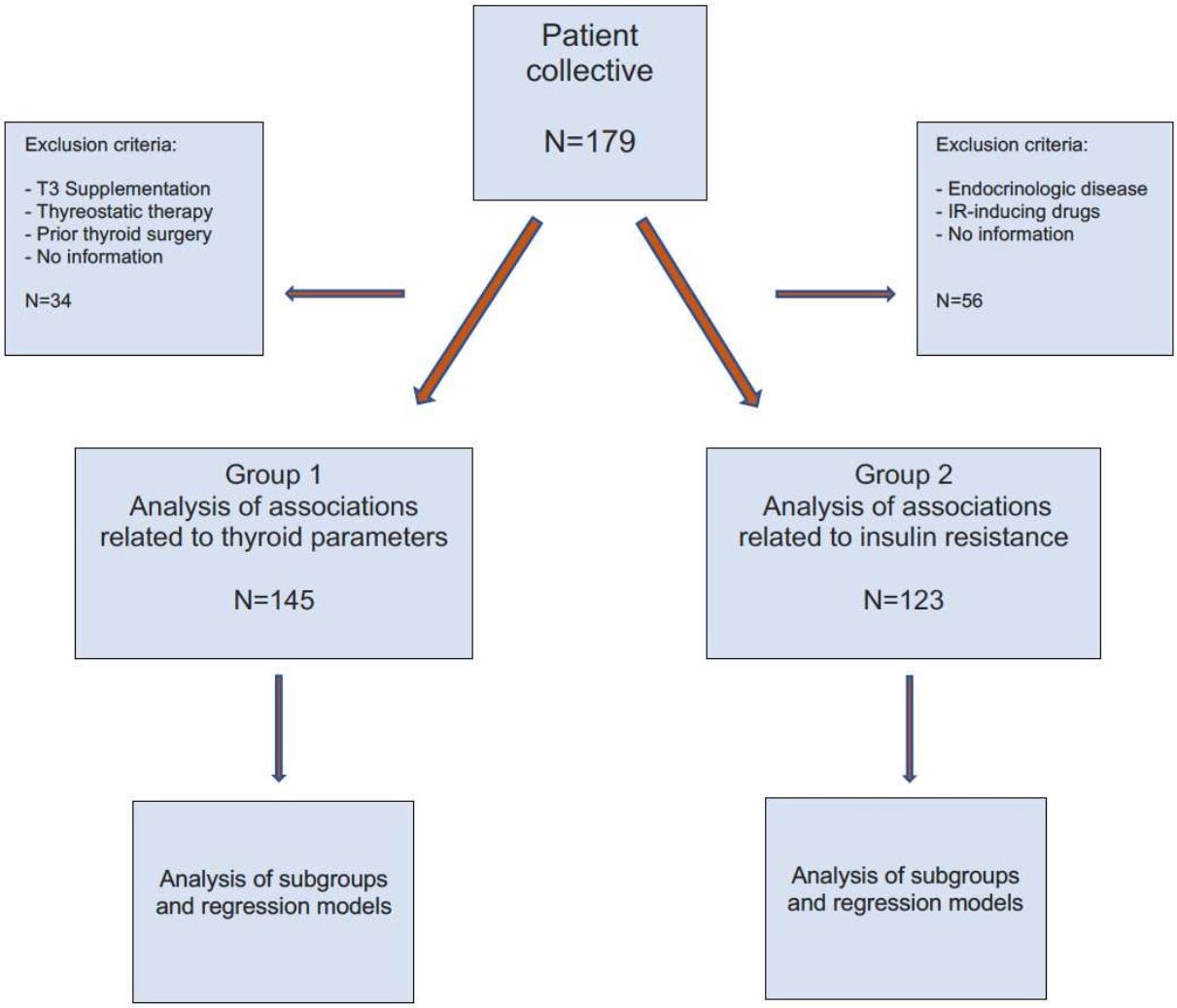

Figure 1. Division of study population for statistical analysis

3.8. Statistical Analysis

For processing and analysis of our data, SPSS Statistics by IBM, Version 28.0.1.1 was used. Normality testing of data has been done by Shapiro Wilk hypothesis testing, interpretation of skewness and kurtosis, and graphical analysis of histograms and Q-Q plots.

Metric variables with distribution according to normality are expressed as means and standard deviation (SD), while metric variables with a high degree of skewness and kurtosis are expressed as medians and quartiles. Categorical variables have been described as frequencies (percentages).

To analyze differences between groups, Student's T-test or Man-Whitney-U test was performed on normally distributed metric data and non-normally distributed metric data, respectively. The effect size of Man-Whitney-U test was calculated as Pearson coefficient "r". Proportions of enumeration data have been compared by chi-square test.

Non-normally distributed variables have been log-transformed for subsequent statistical analyses. We used univariate and multivariate linear regression analysis to show significant relationships between HOMA-IR as dependent variable and thyroid parameters as independent variables in different models.

We also used binary logistic regression and multiple linear regression models to adjust for confounding factors. We checked for outliers of our regression models and ruled out multicollinearity by analyzing VIF and condition indices of the independent variables. Linearity of the logit was checked by method of Box-Tidwell.

Overall significance was set to $p < 0.05$ two-tailed asymptotic.

4. RESULTS

4.1. Description of Whole Study Group

4.1.1. Baseline Characteristics of Subjects

A total number of 179 patients, including 154 women and 25 men, have been admitted to our study. Age distribution was fairly equal, ranging from 18 to 86 years old, with a slight dominance of people under the age of 50 years (57.5%) when compared to people over the age of 50 years (42.5%). 176 participants were German, one Italian, one American, and one of Turkish ethnicity.

Since there was suspicion on certain geographic areas being a risk factor for autoimmune disease, the postcodes of our participants have been examined. All study participants were residing in or in the extended surrounding (up to 130km distance) of Coburg city. Analysis revealed residency of 25.1% of patients in Coburg, 8.9% in Sonneberg, 7.3% in Rödental, and 5.0% in Bad Rodach. The rest of the study group holds residence in smaller cities and villages widely spread over upper Franconia and southern Thuringia, with some outliers in Middle Franconia. Establishing a statistically significant link between patients with autoimmune thyroid disease and specific geographic regions was not possible.

4.1.2. Characteristics Related to Metabolic State

We classified the nutritional status of our patients by the WHO obesity classification, using BMI. Most participants were in a preobese body state, followed by patients who were classified as obesity grade 1, obesity grade 2, and obesity grade 3. Only 19.6% of our study population showed normal BMI. Weight & height information was missing for three patients (N = 3, 1.7%).

We used HOMA-IR to classify our population according to the probability of featuring insulin resistance (Figure 2). We referred to commonly used cut-off values (see Discussion). The majority of patients (33.5%) showed severe insulin resistance with high HOMA-IR values (> 5), similar to occur in patients with diabetes mellitus type 2. While also a considerable part of our population showed a likely chance of having insulin resistance (HOMA-IR 2.5 – 5; 33.0%), for 22.9% an insulin resistance was unlikely (HOMA-IR < 2.0). For another 10.6% an insulin resistance was classified as possible (HOMA-IR 2.0 – 2.5). Concurrently it was concluded that 33.5% of patients had no or only a possible chance of insulin resistance (HOMA-IR < 2.5), while 66.5% had at least a likely chance of altered insulin resistance (HOMA-IR > 2.5).

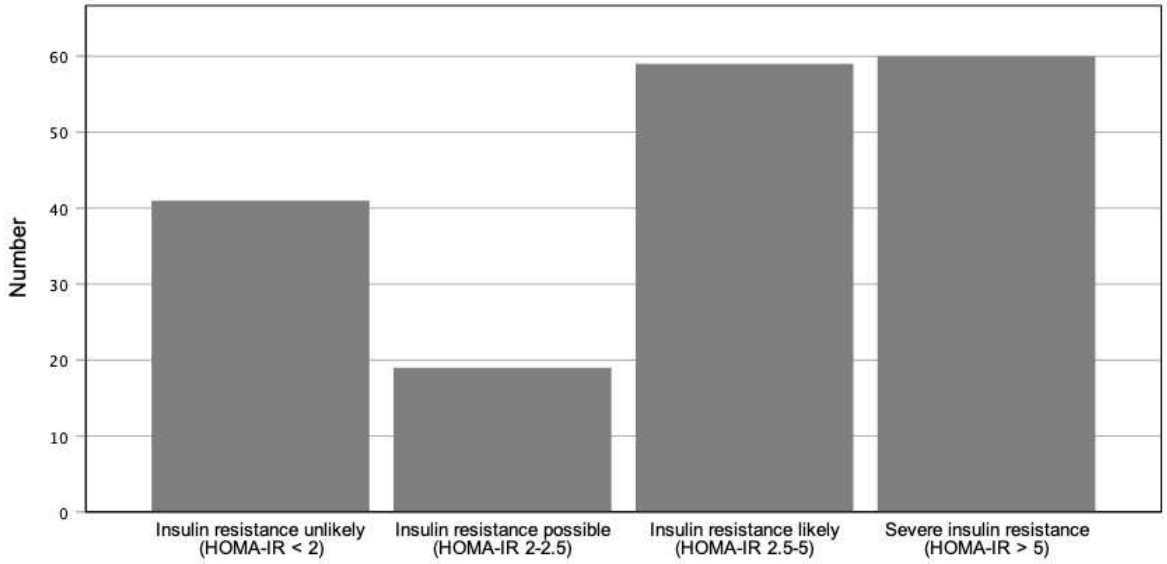

Figure 2. Insulin resistance patterns present across the study population
Data are presented as frequencies

Besides, menopausal status had been defined for all women. The majority of female patients presented in a premenopausal state (66.9%), compared to patients in a postmenopausal state (33.1%).

4.1.3. Lifestyle Habits

By using the self-anamnesis form, we were able to get baseline information on different lifestyle aspects of our participants. A significant part of our study population missed to adequately fill out the prior mentioned anamnesis form. Detailed proportions are as follows:

31.5% stated not consuming alcohol at all, 32.4% consuming alcohol regularly (on several days over the week), and 23.5% consuming alcohol rarely (only on special occasions). Information was missing for 33 patients. We built a new dichotomous variable for our regression analysis and combined people who stated not to consume alcohol at all and people who stated to consume only on special events into “rare alcohol consumption”, as opposed to “regular alcohol consumption”.

61.4% of patients never smoked in their life, 22.8% smoked in the past, and 15.9% are current smokers. The mean of years smoked was 16.8039 ± 9.57292, and the median of packs per day was 0.5 (0.5;1.0). Information was missing for 34 patients. We built a new dichotomous variable only differentiating between people who currently smoke and who don’t.

34.6% of patients had no sleep disturbance, 8.3% had trouble falling asleep and 37.6% had trouble maintaining sleep, 14.5% had a mixture of both. The median sleep duration was 6.5 (6.0; 7.5) hours. Information was missing for 46 patients.

38.5% of participants stated to not perform any sport, 14.0% once per week, 25.2% two to three times per week, and 22.4% performed sport more than three times per week. Information was missing for 36 patients.

4.1.4. Pathologies

Almost half of the sample (N = 75, 41.9%) were people with no prior diagnosed thyroid pathology, while almost the other half of the sample were people with Hashimoto's Thyroiditis (N = 79, 44.1%). Other patients had mostly multinodular goitre (N= 13, 7.3%), Grave's disease (N = 7, 3.9%), thyroid adenoma (N = 3, 1.7%), thyroid autonomy (N = 1, 0.6%) or Thyroiditis de Quervain (N = 1, 0.6%) (see Figure 3).

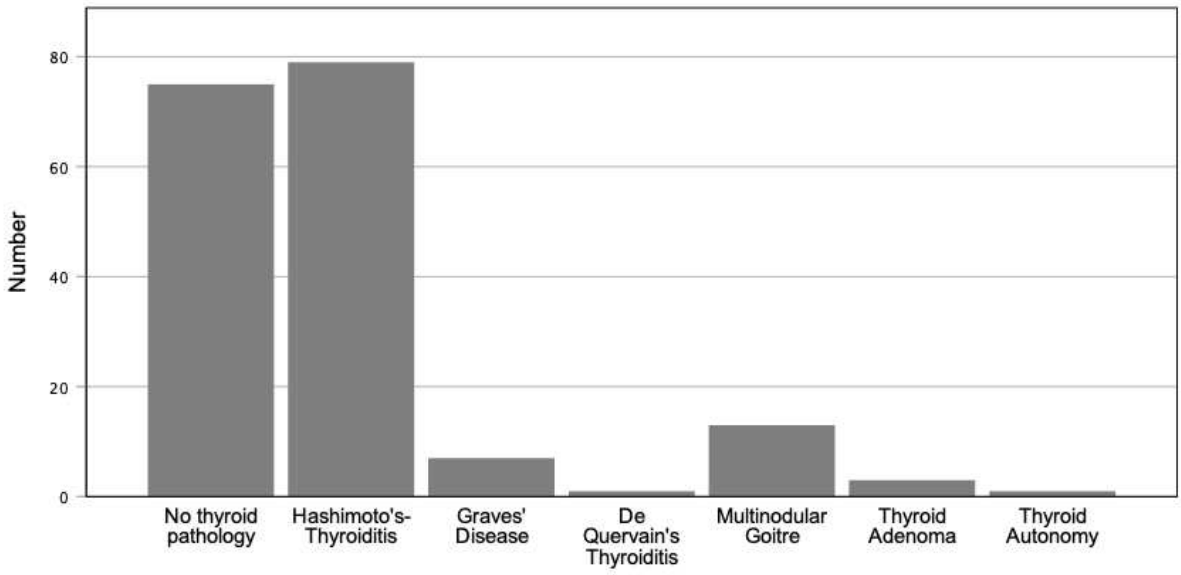

Figure 3. Main thyroid pathologies across the study group
Data are expressed as frequencies

TPOAb levels were used to split patients into autoimmune (N=60) and non-autoimmune groups (N=102). TPOAb laboratory data were missing for some patients (N=17).

83.2% of the population didn't suffer from any other endocrinologic comorbidity, while PCOS (5.6%) and Diabetes Mellitus (5.6%) were other common diagnoses. More rarely, hyperandrogenemia (1.1%), testosterone deficiency (2.2%), partial adrenal insufficiency (0.6%), hypogonadic hypogonadism (0.6%), hypercortisolism (0.6%) or hyperparathyroidism (0.6%) was found.

83.8% of participants involved had no prior diagnosed gastrointestinal comorbidity, 9.5% had gastrointestinal reflux disease, and 3.4% had inflammatory bowel disease. Rarely, patients had a history of gastrointestinal carcinoma (0.6%), gastritis (0.6%), diverticular disease (1.1%), or autoimmune hepatitis (0.6%).

Out of the 179 participants, 1.7% had rheumatoid arthritis in their history, collagenosis (0.6%), fibromyalgia (0.6%), and systemic sclerosis (0.6%) were seen as well.

Arterial hypertension was a diagnosed illness in 24.0% of the study group.

4.1.5. Thyroid Gland Related Characteristics

While most patients (56.4%) have been on current LT4 medication (daily LT4 dose (µg) median was 81.25 (50.0; 100.0)), a combination of LT3 and LT4 therapy was sometimes seen (8.4%). Some were taking thyreostatic medication (2.2%). A big part of our population didn't receive any thyroid drug therapy (32.4%). Information was missing for one participant (0.6%).

Some of our patients have had surgery of the thyroid gland in the past. We distinguished between thyroidectomy (3.9%), partial thyroidectomy (2.8%), and struma resection (3.9%).

Analysis of thyroid function, defined as was mentioned before, revealed Hyperthyroidism in 6.7% of patients, subclinical hyperthyroidism in 5.0%, euthyroidism in 75.4%, 5.6% with subclinical hypothyroidism, 0.6% with hypothyroidism. We could not classify 6.7% of patients according to this classical scheme, due to laboratory value combinations not fitting into the specific ranges. These findings are explainable by an influence of current thyroid medication in these patients. For a visual representation see Figure 4.

We used the extended analysis of thyroid-related parameters by using SPINA-Thyr (see Subjects & Methods). Classification according to "converting type" revealed a dominance of poor converters in our study group (N = 72; 40.2%), followed by moderate converters (N = 63; 35.2%) and good converters (N = 44; 24.6%). The median of TSH Index was 2.7 (2.0; 3.1) ranging from -1.0 to 5.0, while the median of Thyrotroph thyroid hormone resistance index was 123.0 (70.0; 199.0) ranging from 4 to 1572.

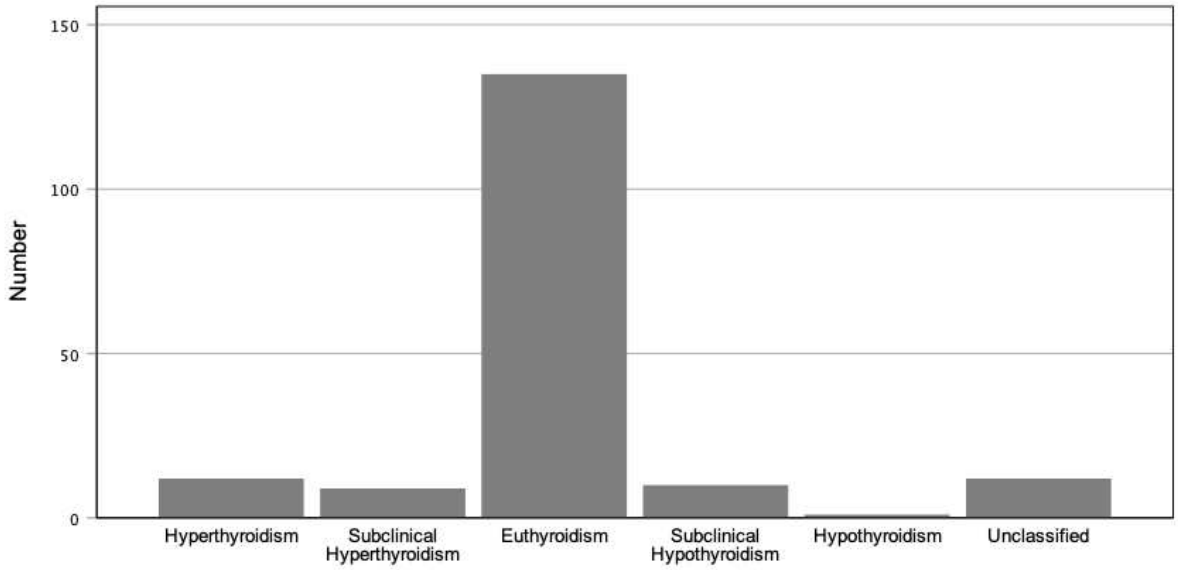

Figure 4. Distribution of thyroid function across the study group
Data are represented as frequencies

4.2. Analysis of Thyroid Parameters

To observe interrelations involving thyroid parameters, we modified the group by additionally excluding patients who were on current fT3 medication, patients who underwent any kind of thyroid surgery in their life, and patients without information on the previously mentioned (see Subjects & Methods).

4.2.1. Subgroup Analysis According to Autoimmune Status

The distribution of fT3 serum level differed significantly between the non-autoimmune (MRank = 76.97) and autoimmune group (MRank = 51.95) ($U = 1322.5$; $Z = -3.665$; $p < 0.001$; $r = 0.318$, as well as the fT3/fT4 ratio between the non-autoimmune group (MRank = 73.99) and the autoimmune group (MRank = 56.45) ($U = 1561.0$; $Z = -2.569$; $p = 0.01$; $r = 0.222$).

A difference in SPINA-GD level between the nonautoimmune (MRank = 74.08) and autoimmune (MRank= 56.32) group could be shown ($U = 1554.0$; $Z = -2.601$; $p = 0.009$, $r = 0.225$). The difference was asymptotic two-sided significant.

Since TPOAbs were used to define the both groups, their distribution was significantly unequal (MRank = 40.50 in the non-autoimmune group vs. MRank = 107.0 in the autoimmune group) ($U = 0.0$; $Z = -9.745$; $p < 0.001$; $r = 0.844$).

TgAbs followed this trend and were signifantly higher in patients with TPOAb positivity (MRank = 85.98) than in patients without TPOAb positivity (MRank = 53.43) ($U = 1061.0$; Z

= -4.905; p= < 0.001; r = 0.426). Extended information about all characteristics can be found in Table 1.

Table 1. Characteristics of study group 1 after stratification into subgroups according to autoimmunity

Parameters		**No thyroid autoimmunity**		**Thyroid autoimmunity**		***P****
N = 145, Missing = 12		*N; M*		*N; M*		
Age (years)		80; 0	46.08 (±12.82)	53; 0	44.91 (±15.64)	0.652†
Gender	Female	70	52.6	45	33.8	0.669‡
	Male	10	7.5	8	6.0	
SBP[a] (mmHg)		79; 1	141.89 (±18.49)	51; 1	136.17 (±15.52)	0.068†
DBP[b] (mmHg)		79; 1	87.0 (80.0; 94.0)	52; 1	84.5 (78.25; 92.0)	0.276
HR[c] (bpm)		78; 2	83.0 (74.0; 95.25)	52; 1	77.0 (72.25; 84.75)	0.047
Height (cm)		79; 1	168.51 (±7.91)	52; 0	167.16 (±8.22)	0.346†
Weight (kg)		79; 1	82.6 (73.7; 97.6)	52; 1	78.7 (67.9; 91.53)	0.097
BMI		79; 1	29.15 (26.26; 34.21)	52; 1	27.91 (24.59; 33.44)	0.119
TSH (µIU/ml)		80; 0	1.37 (0.87; 2.15)	53; 0	1.69 (1.0; 2.87)	0.125
fT3 (pg/ml)		80; 0	3.10 (2.81; 3.36)	53; 0	2.78 (2.52; 3.08)	<0.001
fT4 (ng/dl)		80; 0	1.31 (1.14; 1.53)	53; 0	1.37 (1.19; 1.53)	0.652
fT3/fT4		80; 0	2.39 (1.95; 2.70)	53; 0	2.10 (1.78; 2.50)	0.010
TPOAb (IU/mL)		80; 0	2.0 (0.9; 4.38)	53; 0	70.4 (29.1; 229.15)	<0,001
TgAb (IU/mL)		79; 1	0.9 (0.9; 10.2)	53; 0	17.2 (5.0; 133.6)	<0.001
SPINA-GD[d] (nmol/s)		80; 0	26.35 (21.48; 29.78)	53; 0	23.16 (19.63; 27.2)	0.009
TSHI[e]		80; 0	2.7 (2.0; 3.1)	53; 0	2.8 (2.2; 3.2)	0.263
TTSI[f]		80; 0	131.0 (77.0; 190.75)	53; 0	150.0 (93.5; 225.5)	0.147
LT4 Substitution	Substitution	42	31.6	40	30.1	0.008‡
	No substitution	38	28.6	13	9.8	
LT4 dose (µg)		42; 38	75.0 (50.0; 100.0)	40; 13	75.0 (53.75; 109.0)	0.508
Menopausal status	Premenopausal	46	40.0	27	23.5	0.535‡
	Postmenopausal	24	20.9	18	15.7	

N=number, *M*=missing; Data expressed as means (±standard deviation), medians (quartiles), or as number (%); Significance is given as two-sided asymptotic

* Man-Whitney-U test

† Student's t-test

‡ Chi-square test

[a] Systolic blood pressure

[b] Diastolic blood pressure

[c] Heart rate

[d] Deiodinase activity

[e] TSH Index

[f] Thyrotroph thyroid hormone resistance index

4.2.2. Confounding Factors & Predictor Analysis of fT3

Since we were able to observe a significant decrease in fT3 serum levels in autoimmune thyroid patients, we decided to further elaborate on the low fT3 level and aimed for a consecutive predictor analysis by multiple logistic regression. We used fT3 serum level with cutoff value = 3 pg/ml as a dependent variable and included thyroid autoimmunity, LT4 substitution, BMI, age, and gender as independent variables.

Our model was statistically significant, $\chi^2(5) = 30.141$, $p < 0.001$, resulting in an acceptable amount of explained variance, as shown by Nagelkerke's $R^2 = 0.274$, Cox & Snell $R^2 = 0.206$. The overall percentage of accuracy in classification was 67.2%, with a sensitivity of 75.4% and a specificity of 59.1%. Of the five variables entered into the regression model, three contributed significantly in predicting a fT3 serum level below 3 pg/mL: Thyroid autoimmunity ($p < 0.001$), age ($p = 0.010$), and gender ($p = 0.038$), while LT4 substitution ($p = 0.592$) and BMI showed no significant effect ($p = 0.122$). Therefore, thyroid autoimmunity was identified as the strongest risk factor, increasing the likelihood of presenting fT3 serum levels below 3 pg/mL, OR = 5.227 (95% CI[2.214 -12.339]). Age was also identified as a risk factor, OR = 1.042 (95% CI[1.010 -1.074]), and female gender, OR = 3.886 (95% CI[1.076 – 14.038]). All model coefficients can be found in Table 2.

Table 2. Binary logistic regression analysis; Dependent variable: fT3 serum levels <3 pg/mL

						95% CI	
Predictors	**b**	**SE**	**Wald**	**p**	**Odds Ratio**	**LB**	**UB**
(Constant)	-2.414	1.365	3.126	0.077	0.089		
Age	0.041	0.016	6.704	0.010	1.042	1.010	1.074
Sex	1.357	0.655	4.291	0.038	3.886	1.076	14.038
Thyroid autoimmunity	1.654	0.438	14.243	<0.001	5.227	2.214	12.339
LT4 substitution	0.220	0.412	0.287	0.592	1.247	0.556	2.794
BMI	-0.44	0.029	2.396	0.122	0.957	0.904	1.012

N=131; Cox & Snell R^2=0.206; Nagelkerkes R^2=0.274; $\chi^2(5)$=30.141; p <0.001; Hosmer-Lemeshow Test p=0.921; *Sex: The positive coefficient shows an increase of the likelihood of the dependent variable for female gender

We also performed a multiple linear regression with fT3 serum level as a metric dependent variable and the same independent variables used in the prior logistic regression. Analogously to our logistic model, results also revealed thyroid autoimmunity ($p = 0.013$) and

age (p = 0.002) to be significant predictors, but not LT4 substitution (0.191). Also, gender didn't reach significance as a predictor (p = 0.079) (detailed results not shown).

When we used the same model with log transformed SPINA-GD as dependent variable, we found a significant relationship (F (5,125) = 7.170; p < 0.001) between the SPINA-GD_Ln and LT4 substitution (t (125) = -4.916; p < 0.001). Relationships between SPINA-GD_Ln and age (t (125) = -1.324; p= 0.188), BMI_Ln (t (125) = 1.247; p = 0.215), gender (t (125) = 1.073; p = 0.285), and thyroid autoimmunity (t = -0.806; p = 0.422) have not been significant. Specifically, we found a 21.3% decrease in SPINA-GD_Ln for receiving LT4 substitution therapy (b = -0.213), a 0.125 % increase in SPINA-GD_Ln for every 1% increase in BMI_Ln (b = 0.125), a 0.2% decrease in SPINA-GD_Ln for every one-unit increase in age (b = 0.002), a 6.8% increase in SPINA-GD_Ln for having male gender, and a 3.5% decrease in SPINA-GD_Ln for having autoimmune thyroid disease (b = -0.035).

19.2% of the variance of SPINA-GD_Ln can be explained by the independent variables (adj. R2= 0.192). Therefore, we can conclude that LT4 substitution is the strongest predictor of peripheral deiodinase activity. Details can be found in Table 3.

Table 3. Multiple regression analysis; Dependent variable: SPINA-GD_Ln

						95% CI	
Predictor	**b**	**SE**	**ß**	**t**	**p**	**LB**	**UB**
(Constant)	2.936	0.326		9.005	<0.001	2.291	3.581
Age	-0.002	0.002	-0.106	-1.324	0.188	-0.005	0.001
BMI_Ln	0.125	0.100	0.105	1.247	0.215	-0.073	0.322
Gender	0.068	0.064	0.091	1.073	0.285	-0.058	0.194
Thyroid Autoimmunity	-0.035	0.043	-0.066	-0.806	0.422	-0.120	0.051
LT4 substitution	-0.213	0.043	-0.403	-4.916	<0.001	-0.299	-0.128

N=130; R2=0.223; adj. R2=0.192; F(5; 125)=7.170; p <0.001

4.2.3. Subgroup Analysis According to Menopausal Status

Since gender and age have shown different associations with low fT3 levels and deiodinase activity in our previous models, we further elaborated on this topic. First, we compared premenopausal and postmenopausal females.

There was a two-sided significant difference (U = 1287.5; Z = -2.556 p = 0.011, r = 0.228), between fT3 level in women with premenopausal status (MRank = 69.1) and postmenopausal status (MRank = 51.76). Unlike in the autoimmune vs. nonautoimmune

groups, this difference was found while showing no significant difference in the distribution of SPINA-GD or fT3/fT4 ratio. FT4 serum values remained almost equally between premenopausal (MRank = 63.35) and postmenopausal (MRank = 62.35) women.

Consequently, we performed a univariate linear regression analysis with fT3_Ln as dependent and age as independent variable. The relationship was significant (F (1,143) = 6.056, p = 0.015). 3.4% of the variance of fT3_Ln can be explained by the variable age (adj. R2= 0.034). The regression coefficient of age is -0.002 and is significant (t (143) = -2.384 , p = 0.018). Age is a significant predictor for fT3_Ln. The estimated decrease in fT3_Ln is 0.2% for every one-unit increase in age (b = -0.003 , t (143) = -2.461; p = 0.015). A graphical representation can be found in Figure 5.

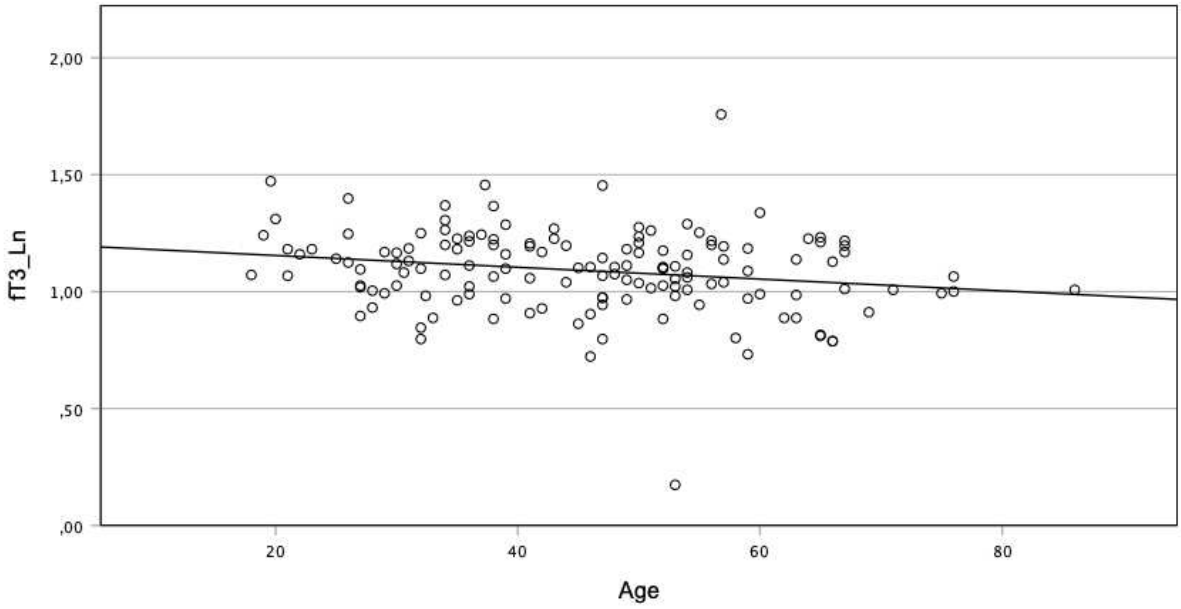

Figure 5. Linear regression analysis of fT3 serum level and age
Age is represented in years, fT3 serum level is given as logarithmically transformed fT3 (pg/mL)

4.2.4. Subgroup Analysis According to Gender

When comparing male and female groups, males (MRank = 91.23) showed a two-sided significantly higher Spina-GD than females (MRank = 70.08) (U = 870.5; Z = -2.134; p = 0.033; r = 0.177), a two-sided significantly higher fT3/fT4 ratio in males (MRank = 91.63) than in females (MRank = 70.02) (U = 863.5; Z = -2.175; p = 0.030; r = 0.181), and a two-sided significant higher fT3 level in males (MRank = 94.55) than in females (MRank = 69.55) (U = 800.0; Z = -2.542; p=0.011; r = 0.211). See Table 4.

Table 4. Subgroup analysis of group 1 according to gender

Parameter	Male (N=20)	Female (N=125)	*P*[*]
fT3 (pg/mL)	3.31 (2.82; 3.44)	2.95 (2.68; 3.29)	0.013
SPINA-GD (nmol/s)	27.76 (23.23; 32.25)	24.76 (20.16; 28.72)	0.037
fT3/fT4	2.53 (2.11; 2.95)	2.24 (1.83; 2.61)	0.033

Data expressed as medians (quartiles); Significance is given as two-sided asymptotic
[*] Man-Whitney-U test

4.2.5. Subgroup Analysis According to Thyroid Hormone Substitution Pattern

Up on comparison of the LT4 substitution groups, a two-tailed significant difference of the fT3/fT4 ratio was obvious, with higher mean in the group without LT4 substitution (MRank=104.49) than in the group on LT4 substitution therapy (MRank= 53.19) (U = 728.5; Z = -7.161; p < 0.001; r = 0.595). A two-sided significant difference (U = 725.5; Z = -7.174; p < 0,001, r = 0.596) was also visible for SPINA-GD, with higher mean in the group without substitution (MRank = 104.54) than in the group on substitution therapy (MRank = 53.15).

Additionally, when comparing the same groups, a two-tailed significant difference (U = 1568.5; Z = -3.751; p < 0.001; r = 0.312) could be shown for higher fT3 levels in the non-substitution group (MRank = 89.49) compared to the substitution group (MRank = 62.62). Results are shown in Table 5.

Table 5. Subgroup analysis of group 1 according to LT4 substitution status

Parameter	No LT4 substitution (N=56)	LT4 substitution (N=89)	*P*[*]
fT3 (pg/mL)	3.13 (2.89; 3.48)	2.88 (2.57; 3.25)	<0.001
SPINA-GD (nmol/s)	29.02 (27.0; 32.32)	22.07 (19.44; 25.27)	<0.001
fT3/fT4	2.65 (2.45; 2.93)	2.01 (1.76; 2.29)	<0.001

Data expressed as medians (quartiles); Significance is given as two-sided asymptotic
[*] Man-Whitney-U test

To further explore the relationship between fT3 serum level and LT4 substitution, we performed a linear regression analysis of fT3_Ln as dependent and LT4 dose_Ln as independent variables. The relationship has not been significant (results not shown).

On the other hand, we also performed a univariate linear regression analysis of SPINA-GD_Ln as dependent and LT4 dose_Ln as independent variables, which has been significant (F (1;87) = 10.581, p = 0.002). 9.8% of the variance of SPINA-GD_Ln can be explained by the variable LT4 dose_Ln (adj. R2 = 0.098). The regression coefficient of LT4 dose_Ln is -0.132 and is significant (t (87) = -3.253; p = 0.002). LT4 dose_Ln therefore is a significant predictor

for SPINA-GD_Ln. The estimated decrease in SPINA-GD_Ln is 0.132% for every 1% increase in LT4 dose_Ln (b = -0.132, t(87) = -3.253; p = 0.002).

4.3. Analysis of Associations Involving Insulin Resistance

To observe different relationships involving insulin & glucose metabolism, we adjusted our original group by excluding patients who were taking drugs known to promote insulin resistance (anti-diabetic drugs, statins, thiazide diuretics, ß-blockers, corticosteroids), patients with any endocrinologic comorbidity, or patients without information on the formerly mentioned (see Subjects & Methods).

4.3.1. Subgroup Analysis According to Autoimmune Status

Neither the distribution of HOMA-IR differed significantly between autoimmune (MRank = 48.26) and non-autoimmune groups (MRank = 60.36) (U = 1110.5; Z = -1.901; p = 0.057; r = 0.181) nor the distribution of insulin between autoimmune (MRank = 49.03) and non-autoimmune groups (MRank = 59.93) (U = 1141.0; Z = -1.714; p = 0.087; r = 0.162) nor the distribution of glucose between autoimmune (MRank = 50.79) and non-autoimmune groups (MRank = 58.94) (U = 1211.5; Z = -1.281; p = 0.2; r = 0.122).

Though, a strong tendency for higher HOMA-IR and insulin levels in the non-autoimmune group was visible, with exact one-tailed significance reaching p = 0.029 and p = 0.043, respectively.

A consecutive chi-square test was used to compare autoimmune status and insulin resistance status. No expected cell frequencies were below 5. Results showed a significance between the groups, $\chi^2(1) = 4.581$, p = 0.032, φ = 0.20. See Table 6 for summarized details.

Table 6. Characteristics of study group 2 after stratification into subgroups according to autoimmunity

Parameter		**No thyroid autoimmunity**		**Thyroid autoimmunity**		***P****
N=123, M=12		*N; M*		*N; M*		
Insulin		71; 0	15.25 (9.84; 29.0)	40; 0	11.34 (7.1; 25.0)	0.087
Glucose		71; 0	91.13 (85.0; 99.69)	40; 0	90.8 (81.55; 95.69)	0.200
HOMA-IR		71; 0	3.40 (2.29; 6.29)	40; 0	2.45 (1.58; 5.81)	0.057
BMI		70; 1	29.10 (26.41; 33.67)	39; 1	27.82 (23.6; 30.23)	0.132
Insulin resistance (IR)	No IR	21	18.9	20	18.0	0.032†
	IR present	50	45.0	20	18.0	

N=number, *M*=missing; Data expressed as medians (quartiles), or as number (%); Significance is given as two-sided asymptotic

* Man-Whitney-U test

† Chi-square test

A more detailed presentation of BMI distribution in these groups is given visually in Figure 6.

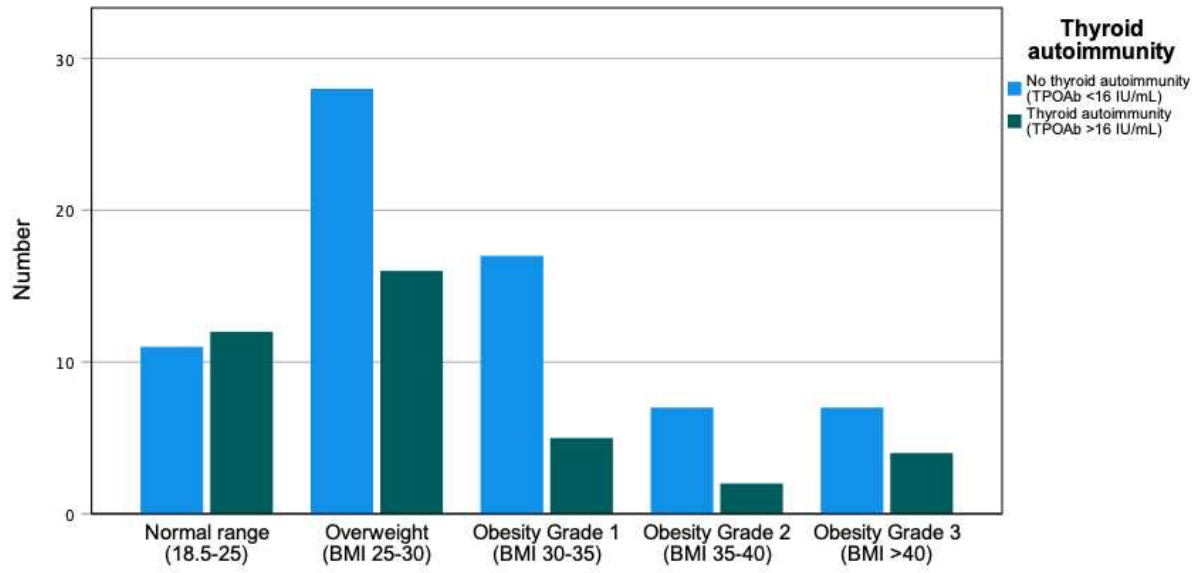

Figure 6. Nutritional status of group 2 participants after stratification according to autoimmune status

4.3.2. Subgroup Analysis According to Gender & Menopausal Status

The distribution of HOMA-IR differed significantly between males (MRank = 81.38) and females (MRank = 59.71) (U = 463.0; Z = -2.073; p = 0.038; r = 0.186). The distribution of insulin between males (MRank = 80.23) and females (MRank = 59.85) followed this trend, but just couldn't reach two sided significance (U = 478.0; Z = -1.95; p = 0.051; r = 0.176). Information on autoimmunity was missing for some males (M = 7), which is why group size was small for men (N = 13).

When observing only females and comparing insulin, glucose, and HOMA-IR levels between premenopausal and postmenopausal groups, no significant differences could be found (results not shown).

4.3.3. Link of Thyroid Parameters and Insulin Resistance

The first step in further observing the relationship between insulin resistance and thyroid disease was to perform regression analyses of HOMA-IR and thyroid parameters.

To adjust for confounding factors, we started by performing a multiple linear regression analysis with HOMA-IR_Ln as a dependent, and age, gender, autoimmune status, BMI_Ln, fT3/fT4_Ln, TSH_Ln, sleep duration_Ln, alcohol consumption pattern, and smoking pattern as independent variables. We were not able to include further possible confounding factors, like

physical activity patterns, certain drug therapies, or nutritional habits into our regression model, due to limited sample size. Information on sleep, alcohol & nicotine was missing for a part of our population, already forcing us to use a smaller sample for this regression analysis. Confounding factors have been identified according to previous literature (93,94,100–102).

Our model was significant for relationships between HOMA-IR_Ln and BMI_Ln, alcohol consumption, and gender. Relationships between HOMA-IR_Ln and age, autoimmune status, and smoking patterns or sleep duration have not been significant. The relationship between HOMA-IR_Ln & fT3/fT4_Ln ratio missed reaching significance closely. Specifically, we found a 0.806% increase in HOMA-IR_Ln for every 1% increase in BMI_Ln, a 49% reduction in HOMA-IR_Ln for not consuming alcohol regularly on several days per week, and a 53.8% increase in HOMA-IR_Ln for having male gender. Out of the non-significant predictors, there was a 0.2% increase in HOMA-IR_Ln for every one-unit increase in age, a 16.5% decrease in HOMA-IR_Ln for having thyroid autoimmune status, a 0.509% increase in HOMA-IR_Ln for every 1% increase in fT3/fT4_Ln, and a 0.237% increase in HOMA-IR_Ln for every 1% increase in smoking pattern. 22.9% of the variance of HOMA-IR_Ln can be explained by the independent variables. Therefore, we can conclude that BMI, alcohol consumption, and gender are the most significant predictors for HOMA-IR. All coefficients and detailed information can be found in Table 7.

Table 7. Multiple linear regression analysis; Dependent variable: HOMA-IR_Ln

						95% CI	
Predictors	**b**	**SE**	**ß**	**t**	**p**	**LB**	**UB**
(Constant)	-2.404	1.212		-1.984	0.051	-4.814	0.006
Age	0.002	0.007	0.034	0.338	0.737	-0.012	0.016
BMI_Ln	0.806	0.373	0.219	2.163	0.033	0.065	1.548
Gender	0.538	0.262	0.201	2.055	0.043	0.017	1.059
Rare alcohol consumption	-0.490	0.165	-0.290	-2.967	0.004	-0.819	-0.162
Non (current) smoker	0.237	0.206	0.110	1.150	0.253	-0.173	0.646
TSH_Ln	0.034	0.071	0.047	0.481	0.632	-0.108	0.176
Sleeping hours_Ln	-0.003	0.003	-0.082	-0.861	0.392	-0.008	0.003
Thyroid autoimmunity	-0.165	0.169	-0.095	-0.975	0.332	-0.500	0.171
fT3/fT4_Ln	0.509	0.296	0.168	1.721	0.089	-0.079	1.098

N=92; R2=0.304; adj. R2=0.229; F(9;83)=4.029; p <0.001

Our next step was to explore the isolated influence of thyroid parameters more in depth, therefore we performed a univariate linear regression with HOMA-IR_Ln as dependent and fT3/fT4_Ln as independent variable, which turned out to be significant (F (1,121) = 8.725, p =

0.004). 6.0% of the variance of HOMA-IR_Ln can be explained by the variable fT3/fT4_Ln. The regression coefficient of fT3/fT4_Ln is 0.830 and is significant (t (121) = 2.954; p = 0.004). FT3/fT4_Ln therefore is a significant predictor for HOMA-IR_Ln. The estimated increase in HOMA-IR_Ln is 0.830% for every 1% increase in fT3/fT4_Ln (b = 0.830 , t (121) = 2.954; p = 0.004). The fT3/fT4_Ln explains a part of the variance of HOMA-IR (adj. R2 = 0.060; F (1;121) = 8.725, p = 0.004). See Figure 7 for a graphical representation. The result stayed significant even after adjustment for BMI_Ln (b = 0.646; t (1; 118 = 2.363; p = 0.020).

Univariate linear regression of HOMA-IR_Ln and SPINA-GD_Ln resulted, analogous to fT3/fT4_Ln in a significant association and could be identified as a positive predictor of HOMA-IR_Ln (results not shown).

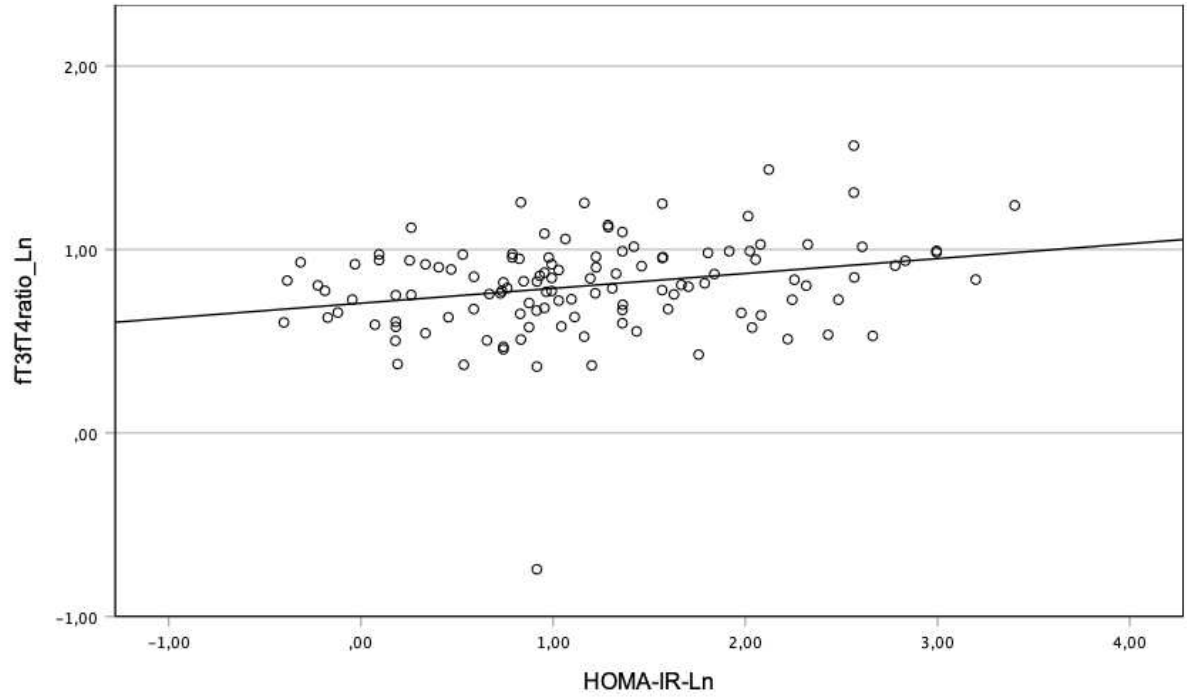

Figure 7. Linear regression analysis of fT3/fT4 and HOMA-IR. HOMA-IR, as well as fT3/fT4, are presented as natural logarithms

To be even more specific about the link of thyroid autoimmunity, thyroid parameters, and insulin resistance, a multivariate regression analysis with HOMA-IR_Ln as dependent, fT3_Ln, fT4_Ln, TSH_Ln and TPOAb_Ln and TgAb_Ln as independent variables has been conducted and was significant between HOMA-IR_Ln and fT3_Ln, and HOMA-IR_Ln and TgAb_Ln. Relationships between HOMA-IR_Ln and TSH_Ln, HOMA-IR_Ln and TPOAb_Ln or HOMA_Ln and fT4_Ln have not been significant. Specifically, we found a 1.194% increase in HOMA-IR_Ln for every 1% increase in fT3_Ln, we found a 0.097% decrease in HOMA-IR_Ln for every 1% increase in TgAb_Ln, a 0.040% increase in HOMA-

IR_Ln for every 1% increase in TSH_Ln, a 0.015% decrease in HOMA_Ln for every 1% increase in TPOAb_Ln and a 0.799% decrease in HOMA_Ln for every 1% increase in fT4_Ln. 10.4% of the variance of HOMA-IR_Ln can be explained by the independent variables. Therefore, we can conclude that fT3 is the strongest predictor of common thyroid parameters towards HOMA-IR. We also can conclude that in our population TgAb titer have been significantly negatively associated with HOMA-IR. All coefficients and detailed data can be seen in Table 8.

Table 8. Multiple linear regression analysis; Dependent variable: HOMA-IR_Ln

						95% CI	
Predictor	**b**	**SE**	**ß**	**t**	**p**	**LB**	**UB**
(Constant)	0.344	0.521		0.661	0.510	-0.689	1.377
TSH_Ln	0.040	0.082	0.054	0.486	0.628	-0.123	0.203
fT3_Ln	1.194	0.441	0.257	2.709	0.008	0.320	2.068
fT4_Ln	-0.799	0.438	-0.196	-1.825	0.071	-1.667	0.069
TPOAb_Ln	-0.015	0.039	-0.039	-0.379	0.706	-0.092	0.062
TgAb_Ln	-0.097	0.042	-0.0234	-2.296	0.024	-0.180	-0.013

N=109; R2=0.145; adj. R2=0.104; F(5; 104)=3.537; p=0.005

Since HOMA-IR is calculated from plasma insulin and glucose values, we were interested in determining which of the parameters correlated best with fT3.

A multiple regression analysis of Glucose_Ln as dependent and TSH_Ln, fT3_Ln, and fT4_Ln as independent variables has not been significant (results not shown).

Contrary to that, in a multivariate regression analysis using Insulin_Ln as dependent variable and the log transformed thyroid parameters as independent variables, we found significant relationships between Insulin_Ln and fT3_Ln, and Insulin_Ln and TgAb_Ln. Relationships between Insulin_Ln and TSH_Ln, Insulin_Ln and TPOAb_Ln, and Insulin_Ln and fT4_Ln have not been significant. Specifically, we found an 1.183% increase in Insulin_Ln for every 1% increase in fT3_Ln, a 0.095% decrease in Insulin_Ln for every 1% increase in TgAb_Ln, an 0.049% increase in Insulin_Ln for every 1% increase in TSH_Ln, a 0.011% decrease in Insulin_Ln for every 1% increase in TPOAb_Ln, and a 0.677 % decrease in Insulin_Ln for every 1% increase in fT4. 10.6% of the variance of Insulin_Ln can be explained by the independent variables. Therefore, we can conclude that fT3_Ln is a positive significant predictor for Insulin_Ln, while TgAb titer is a negative significant predictor. Exact values can be found in Table 9.

Table 9. Multiple linear regression analysis; Dependent variable: Insulin_Ln

						95% CI	
Predictor	**b**	**SE**	**ß**	**t**	**p**	**LB**	**UB**
(Constant)	1.799	0.492		3.659	<0.001	0.824	2.774
TSH_Ln	0.049	0.078	0.070	0.628	0.531	-0.105	0.202
fT3_Ln	1.183	0.416	0.270	2.843	0.005	0.358	2.008
fT4_Ln	-0.677	0.413	-0.176	-1.638	0.105	-1.496	0.143
TPOAb_Ln	-0.011	0.037	-0.031	-0.303	0.763	-0.084	0.062
TgAb_Ln	-0.095	0.040	-0.242	-2.381	0.019	-0.174	-0.016

N=109; R2=0.147; adj. R2=0.106; F(5; 104)=3.596; p=0.005

5. DISCUSSION

Our study investigated the association between thyroid parameters and insulin resistance in patients with and without autoimmune thyroid disease. The results could show that fT3 serum level is a positive predictor for insulin serum level, HOMA-IR, and therefore insulin resistance (as delineated by our regression models, see Table 8, Table 9). These findings confirm current research on other populations on this topic (17,19,116). Additionally, we could show that fT3/fT4 and SPINA-GD are positively associated with insulin serum levels and HOMA-IR and that they are more significant markers than TSH and fT4 serum levels for insulin resistance. Therefore, we could confirm the results of several studies, including a huge study with 132346 participants (117) and a study with 26719 participants, showing association of increased fT3 levels and components of metabolic syndrome (84). However, it is not clear if these observations are built on the concept of thyroid hormones (fT3) directly interfering with glucose and insulin metabolism or indirectly influencing body fat composition and subsequently affecting insulin resistance. To increase insulin secretion, thyroid hormones could be directly stimulating pancreatic islet (ß) cell maturation and function, as has been shown before by Matsuda *et al.* in studies on the zebrafish, and as has been discussed in some other recent human studies (118,119,119–122). On the other hand, it might be possible that the excess of nutrition in obese patients could trigger an increase in deiodinase activity to make more fT3 available in order to adapt for nutritive overload in these patients, by way of stimulating thermogenesis and metabolic processes (84,123). Further research is necessary to evaluate causative relationships more in-depth.

Our findings on the correlation between thyroid autoimmunity and insulin resistance are more suspicious to raise some questions. We could not find two-tailed significant differences in insulin resistance, insulin serum levels, or glucose serum levels between patients with and without autoimmune thyroid disease. We did find one-tailed significant differences, referring to lower insulin resistance, lower HOMA-IR, and lower insulin levels in patients with autoimmune thyroid disease. When we performed our regression analyses with HOMA-IR as a dependent and thyroid autoantibodies as independent variables, the regression coefficients showed a negative association, reaching significance for TgAb. This is neither consistent with recent research, which identified a higher prevalence of metabolic syndrome in autoimmune thyroid patients (81), nor with literature associating obesity with high TPOAbs (21), nor literature showing a linear increase in TPOAbs with HOMA-IR (20).

Our multiple regression model identified BMI to be a much stronger predictor for insulin resistance than thyroid parameters (as is broadly established knowledge (124–127)), what most probably could be the explanation for our findings since BMI was generally higher in the non-

autoimmune group (even if not significant). Besides BMI, we determined regular alcohol consumption and male gender to be significant predictors of HOMA-IR. Still, IR has even more possible confounders with much stronger effects than thyroid metabolism, like waist circumference, body fat%, nutritional patterns, or physical activity patterns.

For future research on this topic, it would be advisable to collect more detailed information about exact body measures, lifestyle habits, and metabolic disturbances commonly found in overweight patients or patients with metabolic syndrome to be able to eliminate or adjust all confounders and get a more isolated picture of the effect of thyroid metabolism and to avoid bias.

Our secondary findings of observing higher HOMA-IR and insulin values in male patients have to be taken cautiously due to small sample size (N=13), although they are in accordance with already known gender differences regarding insulin & glucose metabolism (128,129).

We couldn't find significant differences in insulin resistance between pre-and postmenopausal patients. Still, insulin and HOMA-IR tended to be lower in the postmenopausal group, which could be in accordance with our observations of lower fT3 levels in postmenopausal women. These findings are also following recently published literature with bigger sample sizes observing a similar effect in their study group (18). On the contrary, a well established concept is the increase in insulin resistance (mediated by skeletal muscle aging) and diabetes mellitus rates with increase in age, as has been shown by many studies before (130–134).

When analyzing thyroid parameters, our findings revealed that serum fT3 levels, Spina-GD, and fT3/fT4 are significantly lower in patients with autoimmune thyroid disease. These findings are in accordance with a recent meta-analysis, confirming abnormal fT3 and TSH serum levels in patients with AITD (135). Since fT3 is mainly produced by the conversion of fT4, it is largely dependent on the action of peripheral deiodinases. Recent studies focused on different influences on the conversion activity of deiodinases and proposed possible disturbances of these processes by various factors, like age, BMI, or LT4 treatment (22). We therefore decided to explore our findings in more detail since they could be related to our main hypothesis and altering insulin resistance.

Ultimately, we were not able to prove linear correlations between fT3 serum levels and thyroid autoantibody titers. Linear regression models have shown no significant results. Still, the difference in fT3 serum level is significantly lower in patients with general TPOAb

positivity, as was shown by our direct comparisons of autoimmune vs. non-autoimmune groups (see Table 1).

On the other hand, our study could identify the significantly higher prevalence of low fT3 serum levels, low SPINA-GD, and low fT3/fT4 in patients on LT4 substitution therapy (Table 5), while at the same time, there were no significant differences in BMI distribution between patients with and without LT4 substitution (results not shown). Additionally, we could observe a decrease in deiodinase conversion capacity with an increase in LT4-substitution doses. These findings are in accordance with larger studies performed by Hoerman *et al.* (14,22,112). We could not find any statistical significance when observing the relationship between LT4 substitution doses and fT3 serum levels (results not shown).

Our logistic regression models showed thyroid autoimmunity to be the strongest predictor of fT3 serum levels below 3 pg/ml, while for peripheral deiodinase activity, LT4 substitution was identified as the strongest predictor (see Table 2 and Table 3, respectively).

Further than that, our predictor analyses identified an increase in age and female sex as risk factors for fT3 serum levels below 3 pg/mL, but not for a reduced deiodinase activity. This implicates a significant role of other factors. This suspicion was confirmed when directly observing pre-and postmenopausal women. We found a significant difference in fT3 serum level, but not in SPINA-GD. In addition, this effect was lost when only splitting the groups into younger and older than 50 years and including men. In summary, our findings indicate that there is a high likelihood of a modifying role of female sex hormones on fT3 serum levels.

We could also find higher fT3 serum levels under higher SPINA-GD and fT3/fT4 ratios in males than in females. Although this finding might not be representative due to our small sample size of male patients (N=20), it supports the former suspicion. These findings align with observations on the occurrence of hypothyroidism after menopause or thyroid stimulatory effects of female sex hormones (136,137). Additionally, we confirmed a linear relationship between fT3 and age, pointing out that fT3 serum levels are decreasing with an increase in age, as has been shown before (138).

A very recent analysis from Germany, proposed age to be an important overlooked factor in past studies and suggested that age could have an influential role in the relationship between thyroid function and insulin resistance (19).

When interpreting the results of our study, some limitations have to be taken into account. Since this is a cross-sectional study, no causal relationships could be shown. Therefore, there is a need for future prospective studies to verify these relationships. Furthermore, this study was conducted mainly in a population with a single ethnic (German) background. It,

therefore, cannot be generalized internationally. Moreover, since mostly females were included in the study, results cannot be generalized to both sexes, either. In addition, the power of this study would profit from a bigger sample size.

Some general problems in using specific definitions to describe our study population and associations between groups became obvious.

Firstly, for this study thyroid autoimmunity was defined by the presence of TPOAbs > 16 IU/ml, which could well be a methodological weakness of definition, as the role and presence of antibodies in the pathogenesis of the disease are currently under discussion. Thyroid autoantibodies are only positive in 90-95% of patients with autoimmune thyroid disease, and they are not measurable in all phases of the disease (139).

This former definition might therefore miss a potentially significant part of patients who suffer from AITD but have negative autoantibody status. In addition, there is no standardized opinion on which antibody titers are pathological and which could appear in normal physiologic conditions and reference ranges vary.

Secondly, we could not analyze all possible confounding factors for insulin resistance. For example, we have had insufficient information on exact body measures, regular alcohol consumption, smoking patterns, sleeping patterns, patterns of daily physical behavior, and detailed nutrition habits. Furthermore, since sample size was limited we decided to not include all possible factors in our regression models.

Thirdly, patients on current thyroid medication made up a considerable part of our sample. Accordingly, there might be cases with an inadequate display of thyroid parameters in laboratory results due to over or under substitution, or delayed responses to therapy, e.g., commonly known for TSH.

Fourthly, the inclusion of study participants was mainly based on available HOMA-IR laboratory values. Common indications for the determination of HOMA-IR are suspicion of insulin resistance, Diabetes Mellitus Type 2, Metabolic Syndrome, or PCO-Syndrome. Even after exclusion of common endocrinologic comorbidities in our analyses, our study population tended to be in obese body states, therefore being biased towards having higher HOMA-IR values and a lot of possible confounders.

Fifthly, certain issues with the definition of insulin resistance by HOMA-IR itself arise. Common reference ranges are partly based on ROC curves, partly on percentiles, and reference ranges are not adapted towards age, gender, ethnical background, or different insulin and glucose assays (140). On top of that, HOMA-IR mostly represents central (liver) resistance to insulin, while other methods, especially the TyG index, represent peripheral (muscle) resistance

(18). In summary, this lack of standardization and generalization of methods aggravates the difficulty of understanding processes involved in thyroid, insulin, and glucose metabolism, especially by cross-sectional study designs. It limits the power and comparability in general medical research.

Despite mentioned limitations, we have to emphasize that we did put effort into focusing on possible confounders, an issue often neglected by other studies. For analyses of insulin resistance-associated interrelations, we excluded patients with a vast variety of IR-promoting drugs (not only anti diabetics, but also ß-blockers, statins, thiazide diuretics, corticosteroids, etc.) and also excluded patients with endocrinologic comorbidities that could well influence insulin resistance, like PCOS or testosterone deficiency. In addition, the age of our study group was well balanced, ranging from 18 to 86 years old, giving a good general picture of examined relationships. We also have to emphasize to be the first study observing these associations in an Upper Franconian/Southern Thuringian population. Since this area features the highest incidences of thyroid autoimmune diseases (unpublished data) in Bavaria, examining this population is of special interest.

6. CONCLUSIONS

The results of this study showed a positive association between insulin resistance and fT3 serum levels, fT3/fT4 ratio, and SPINA-GD, independent of thyroid autoantibody status. We could show that fT3 is associated with insulin, not glucose serum levels. We indicated the relationship of lowered insulin resistance parameters in our AITD group, thereby exploring possible weaknesses of our study and setting the focus on the importance of confounding factors. Increased BMI, regular alcohol consumption, and male gender have been identified as the strongest predictors for the presence of insulin resistance in our study population.

Our outcomes also point out that patients of mixed age and different nutritional statuses with positive TPOAb status are associated with low fT3 serum levels. In contrast, patients on LT4 substitution therapy do present with lower conversion activity of peripheral deiodinases. Furthermore, we could show the inverse relationship between age and fT3 levels, as well as the correlation of female sex with lower fT3 levels.

Paying more attention to these relationships could help explain some of the difficulties we encounter today in treating AITD and insulin resistance.

There is a need to assess and reduce the risk of altered insulin resistance since consequences have a severe impact on possible chronic complications in any patient, especially in patients with concomitant thyroid disease.

In coherence, our study emphasizes the need to closely monitor fT3 level alterations in patients with autoimmune thyroid disease and patients under LT4 substitution since they are at the most considerable risk of having thyroid hormone conversion issues.

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8. SUMMARY

Objectives: Our main goal was to observe associations between laboratory thyroid parameters free triiodothyronine (fT3), free thyroxine (fT4), thyroid-stimulating-hormone (TSH), thyroid peroxidase antibodies (TPOAbs), thyroglobulin antibodies (TgAbs), deiodinase activity (SPINA-GD), thyrotroph thyroid resistance index (TTSI), thyroid stimulating hormone index (TSHI), and the homeostatic model assessment of insulin resistance (HOMA-IR). We were particularly interested in comparing these relationships in patients with and without autoimmune thyroid disease.

A secondary intention was to examine possible influences connected to the prevalence of low fT3 serum levels in our patient collective, especially due to our suspicion of fT3 serum level alterations in autoimmune thyroid patients.

Patients and methods: This cross-sectional study was performed retrospectively in a patient collective of 179 mostly German participants, featuring 25 males and 154 females. The distribution of thyroid autoimmune status vs. non-autoimmune status was fairly equal. Most patients have been in a euthyroid state, a large part under current LT4 substitution therapy. We directly compared differences in laboratory parameters in our different subgroups and also examined linear relationships by the use of regression models.

Results: Two-tailed significant differences could be shown for lower serum fT3 levels, lower SPINA-GD, as well as lower fT3/fT4 in autoimmune thyroid patients. We also observed lower fT3 levels in postmenopausal females and showed the linear inverse relationship between fT3 and age. We were able to identify BMI, regular alcohol consumption, and male gender as significant predictors for higher HOMA-IR values in our study population. Furthermore, we showed linear relationships between fT3 serum levels and insulin as well as and HOMA-IR. We also identified LT4 substitution therapy as being the main negative predictor for SPINA-GD.

Conclusion: Thyroid autoimmune disease is a significant predictor for the prevalence of fT3 serum levels below 3 pg/ml, while LT4 substitution therapy is a significant predictor for the reduced activity of peripheral deiodinases. FT3 serum level is the most significant predictor of basic thyroid parameters towards HOMA-IR and insulin levels. Targeted prospective studies need to follow to evaluate on associations found in our study.

9. CROATIAN SUMMARY

Ciljevi: Naš glavni cilj bio je promatrati povezanost između laboratorijskih parametara štitnjače slobodni trijodtironin (fT3), slobodni tiroksin (fT4), hormon koji stimulira štitnjaču (TSH), antitijela na peroksidazu štitnjače (TPOAbs), antitijela na tireoglobulin (TgAbs), aktivnost dejodinaze (SPINA-GD), tireotrof indeks otpornosti štitnjače (TTSI), indeks hormona stimulirajućeg štitnjače (TSHI), i homeostatskog modela procjene inzulinske rezistencije (HOMA-IR). Posebno nas je zanimala usporedba tih odnosa u bolesnika s i bez autoimune bolesti štitnjače. Sekundarna namjera bila je ispitati moguće utjecaje povezane s prevalencijom niske razine fT3 u serumu kod naše skupine pacijenata, posebno zbog sumnje na promjene razine fT3 u serumu u autoimunih bolesnika štitnjače.

Pacijenti i metode: Ova presječna studija provedena je retrospektivno na skupini od 179 pacijenata, uglavnom njemačkih sudionika, uključujući 25 muškaraca i 154 žene. Distribucija autoimunog statusa štitnjače u odnosu na ne-autoimuni status bila je prilično jednaka. Većina pacijenata bila je u eutireoidnom stanju, velikim udjelom pod supstitucijskom terapijom LT4 u tom trenutku. Izravno smo usporedili razlike laboratorijskih parametara naših različitih podskupina te smo također ispitali linearne odnose korištenjem regresijskih modela.

Rezultati: Mogle su se vidjeti dvostrane značajne razlike kod nižih razina fT3 u serumu, niži SPINA-GD, kao i niži fT3/fT4 u pacijenata s autoimunom bolesti štitnjače. Također smo primijetili niže razine fT3 kod žena u postmenopauzi i pokazali smo linearni inverzni odnos između fT3 i dobi. Uspjeli smo izdvojiti BMI, redovitu konzumaciju alkohola i muški spol kao značajne predispozicije za više vrijednosti HOMA-IR u našoj studijskoj populaciji. Nadalje, pokazali smo linearne odnose između serumskih razina fT3 i inzulina kao i HOMA-IR. Također smo izdvojili supstitucijsku terapiju LT4 kao glavnu negativnu predispoziciju za SPINA-GD.

Zaključci: Autoimuna bolest štitnjače značajan je predznak prevalencije serumskih razina fT3 ispod 3 pg/ml, dok je supstitucijska terapija LT4 značajan predznak smanjene aktivnost perifernih dejodinaza. Razina fT3 u serumu najznačajniji je predznak osnovnih parametara štitnjače prema razinama HOMA-IR i inzulina. Ciljane studije o mogućnostima bi trebale slijediti kako bi se procijenile povezanosti pronađene u našoj studiji.

10. CURRICULUM VITAE

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Educational background

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