Diploma thesis

MicroRNA-signatures in autoimmune thyroid disease

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Graz, 01.04.2023

Statutory Declaration

I hereby declare that I have authored the present diploma thesis independently and without external help, that I have not used other than the declared sources and that I have explicitly marked all material and sections that have been quoted, either literally or by content.

Graz, 01.04.2023 *Edi Arifi m.p.*

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List of abbreviations

Ab	antibody
Ago	Argonaute protein
AITD	autoimmune thyroid disease
cDNA	complementary DNA
Cq	quantification cycle
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CVD	cardiovascular disease
DGCR8	DiGeorge syndrome critical region 8
ERK 1/2	extracellular signal-regulated kinases 1 and 2
FoxP3	forkhead box P3
GD	Grave's disease
HDL	high density lipoprotein
HT	Hashimoto's thyroiditis
IFN-γ	interferon γ
IGF-1	Insulin-like growth factor 1
IgG	immunoglobulin G
LDL	low density lipoprotein
LT4	levothyroxine
MCT	monocarboxylate transporter
miRNA	microRNA
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NIS	Na^+/I^+ symporter
OATP1C1	organic anion transporter 1 C1
PBMC	peripheral blood mononuclear cells
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3ctl kinase
Pre-miRNA	precursor miRNA
Pri-miRNA	primary miRNA
PTPN22	protein tyrosine phosphatase non-receptor type 22

qRT-PCR	quantitative real time polymerase chain reaction
Ran-GTP	GTP-binding nuclear protein Ran
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rT3	reverse T3
scH	subclinical hypothyroidism
SDF	source data form
T3	triiodothyronine
T4	thyroxine
TG	thyroglobulin
TG-Ab	thyroglobulin antibody
TH	thyroid hormone
Th1	T helper cell type 1
Th2	T helper cell type 2
Th17	T helper cell type 17
TNF-α	tumor necrosis factor α
TNRC6	trinucleotide repeat-containing gene 6A protein
TPO	thyroid peroxidase
TPO-Ab	TPO antibody
TRAb	TSH receptor antibody
Treg	regulatory T cell
TRH	TSH releasing hormone
TSH	thyroid stimulating hormone
UTR	untranslated region

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Zusammenfassung

Hintergrund

Autoimmune Schilddrüsenerkrankungen sind die häufigsten Autoimmunerkrankungen und stellen in Industrieländern den häufigsten Grund für eine Hypothyreose dar. Die Substitution mit Schilddrüsenhormonen ist eine der meistverschriebensten Therapien.
MicroRNAs sind kurze nicht-kodierende RNAs, die sich intrazellulär und im Blutkreislauf nachweisen lassen, und die Translation modulieren. Sie sind an diversen physiologischen und pathologischen Prozessen beteiligt und stehen im Zusammenhang mit zahlreichen Krankheitsbildern wie etwa Autoimmunerkrankungen und Schilddrüsenerkrankungen.

ProbandInnen und Methoden

In der Ambulanz der Abteilung für Endokrinologie wurden 46 Proband:innen rekrutiert. Es wurde bei 8 Patient:innen mit Autoimmunthyreopathie Hashimoto und 18 gesunden Kontrollen ein Panel aus 9 zirkulierenden miRNAs (miR-21-5p, -22-3p, -22-5p, -96-5p, -142-3p, -146a-5p, -301-3p, -375, -451) mittels qRT-PCR analysiert, die bei autoimmunen Schilddrüsenerkrankungen in vorhergehenden Studien eine veränderte Expression gezeigt haben.

Ergebnisse

In unserer Studienkohorte ließ sich kein signifikanter Unterschied in der miRNA-Expression zwischen den gesunden Kontrollen und Patient:innen mit Immunthyreopathie Hashimoto nachweisen. Allerdings zeigte sich bei miR-22-3p innerhalb der Hashimoto-Gruppe eine auffällige Verteilung der Expressionsmuster, die auf zwei unterschiedliche Subgruppen bei HT hinweisen könnte. Hier zeigen sich in den zwei Subgruppen unterschiedlich hohe Antikörpertiter, obwohl aufgrund der kleinen Fallzahl keine Signifikanzbestimmung möglich ist.

Diskussion

Zusammenfassend lässt sich der in vorausgegangen Studien nachgewiesene Unterschied in der miRNA-Expression nicht replizieren. In zukünftigen miRNA-Studien bei AITD sollte ein Augenmerk auf miR-22-3p gelegt werden, um dessen klinischen Nutzen festzustellen.

Abstract

Background

Autoimmune thyroid diseases are the most common autoimmune disorders and the most common cause of hypothyroidism in developed countries. The substitution of thyroid hormones is one of the most prescribed therapies.

MicroRNAs are short, non-coding RNAs that can be detected intracellularly or in the circulation and that modulate translation. They are involved in various physiological and pathological processes and are linked to numerous disorders such as autoimmune or thyroid disease.

Patients and methods

46 participants were recruited in the outpatient clinic of the Division of Endocrinology and Diabetology. A panel of 9 circulating microRNAs (miR-21-5p, -22-3p, -22-5p, -96-5p, -142-3p, -146a-5p, -301-3p, -375, -451) was analyzed in 8 patients with Hashimoto's thyroiditis and 18 healthy controls using qRT-PCR based on previous studies on miRNAs in autoimmune thyroid disease.

Results

The study cohort showed no significant difference in microRNA expression between patients with Hashimoto's thyroiditis and healthy controls. However, miR-22-3p showed a conspicuous distribution in the Hashimoto group suggestive of two distinct subgroups that can be found in HT. The subgroup with low miR-22-3p expression showed lower levels of thyroid antibodies although the sample size is too small to calculate the significance.

Discussion

In summary the previously detected difference in miRNA expression could not be replicated. In future studies on miRNAs in AITD further analysis on miR-22-3p should be included to assess its' clinical utility.

Information on previously submitted publications

In January 2022 Olivia Trummer, Ines Foessl, Natascha Schweighofer, Edi Arifi, Christoph W. Haudum, Sharmaine Reintar, Stefan Pilz, Verena Theiler-Schwetz, Christian Trummer, Andreas Zirlik, Albrecht Schmidt, Caterina Colantonio, Ewald Kolesnik, Nicolas Verheyen, Thomas R. Pieber and Barbara Obermayer-Pietsch published "*Expression Profiles of miR-22-5p and miR-142-3p Indicate Hashimoto's Disease and Are related to Thyroid Antibodies*" in *Genes*.

Abstract: Hashimoto's thyroiditis (HT) is the most prevalent autoimmune disorder of the thyroid (AITD) and characterized by the presence of circulating autoantibodies evoked by a, to date, not fully understood dysregulation of the immune system. Autoreactive lymphocytes and inflammatory processes in the thyroid gland can impair or enhance thyroid hormone secretion. MicroRNAs (miRNAs) are small noncoding RNAs, which can play a pivotal role in immune functions and the development of autoimmunity. The aim of the present study was to evaluate whether the expression of 9 selected miRNAs related to immunological functions differ in patients with HT compared to healthy controls. MiRNA profiles were analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 24 patients with HT and 17 healthy controls. Systemic expressions of miR-21-5p, miR-22-3p, miR-22-5p, miR-142-3p, miR-146a-5p, miR-301-3p and miR-451 were significantly upregulated in patients with HT ($p \leq 0.01$) and were suitable to discriminate between HT and healthy controls in AUC analysis. Altered expressions of miR-22-5p and miR-142-3p were associated with higher levels of thyroid antibodies, suggesting their contribution to the pathogenesis of HT.(3)

1 Introduction

Autoimmune thyroid diseases (AITD) are highly common diseases in Europe, affecting about 5% of the general and up to 10% of the female population.(4) AITD are also considered to be the most common autoimmune diseases.(5) Levothyroxine is prescribed to about 3% of the population in certain countries, making it one of the most prescribed drugs in developed countries.(6)

This high prevalence grants AITD a special position in the domain of otherwise rather rare autoimmune disorders. Therefore, a deeper understanding of the pathogenesis, cellular and humoral changes in this autoimmune thyroid disorder could help gain a further understanding of autoimmune diseases in general. In addition to the high prevalence, AITDs have a unique position, as they are not treated by immune modulation, but hormone substitution only. Hence, offering an otherwise unregistered view on an autoimmune disease not masked by immunosuppressive therapy.

MicroRNAs (miRNAs) are part of many small RNA molecules found in the last two decades. Their high level of conservation throughout species seems to pinpoint at a biological relevance that is yet to be fully understood. These miRNAs share their function of modulating the protein biosynthesis around thyroid hormones and their mode of transport, namely in the blood, with autoimmune disorders, therefore making them a prime target for investigations in this context.

The aim of this study is to investigate the relationship between specific miRNAs that have shown differential expression in previous studies. In addition to pathophysiological insights miRNAs are examined for their diagnostic value as modern biomarkers in thyroid disorders.

2 Background

2.1 Thyroid gland

2.1.1 Anatomy

The thyroid gland is an endocrine organ located in the anterior lower neck. It wraps around the anterior trachea and is located just below the larynx. Two lobes make up the bulk of the gland and are connected by a narrow strip of glandular tissue called the “isthmus”. The two lobes have a total volume of up to 25mL in males and 18mL in females. Under physiologic conditions they are neither visible nor palpable. In about 50% of humans, a small accessory lobe, the “pyramidal lobe”, reaches from the isthmus cranially towards the hyoid bone and foramen cecum, a small depression at the root of the tongue.

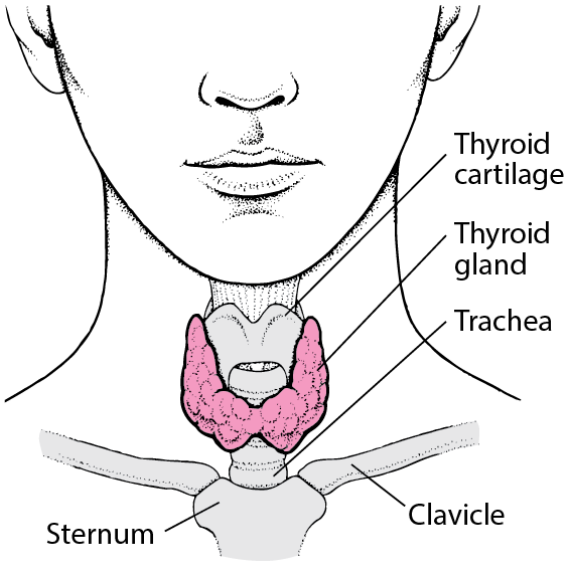


Figure 1 Thyroid gland(1)

The thyroid is surrounded by two capsules: The delicate internal capsule sends septa into the stroma and separates the lobes into smaller lobules. The fibrous external capsule covers the thyroid and connects it to the trachea and surrounding tissue. On the dorsal side of each thyroid lobe, but still within the fibrous capsule, lie four parathyroid glands. These glands functionally form a distinct gland that produces parathyroid hormone which increases serum calcium levels.

The fibrous capsule is considered part of the deep cervical fascia which also surrounds trachea and esophagus. On the lateral side of the thyroid, this fascia forms the carotid sheath, fibrous connective tissue that surrounds the common carotid artery, parts of the internal carotid artery, the internal jugular vein and the vagus nerve.

Thyroid blood supply is run by two pairs of arteries. The superior thyroid artery branches off from the external carotid artery and enters the fibrous capsule at the superior thyroid pole supplying the ventral side of the gland. The inferior thyroid artery arises from the thyrocervical trunk and enters the fibrous capsule on the inferior pole supplying the dorsal

side. The thyroid "*ima"* artery can be found in about 10% of humans. It either arises from the brachiocephalic trunk or the aortic arch and ascends to the isthmus.
The venous drainage is driven by three pairs of veins. The superior thyroid vein accompanies the superior thyroid artery and drains into the internal jugular vein together with the middle thyroid vein. The inferior thyroid vein follows the path of the inferior thyroid artery and drains into the brachiocephalic vein.

The vagus nerve operates the sensible and parasympathetic innervation of the thyroid gland through the superior laryngeal nerve and the recurrent laryngeal nerve. The latter diverges in the mediastinum and wraps around the subclavian artery one the right side while the left one wraps around the aortic arch. Both then reach the esophagus and trachea and follow their path up back to the thyroid gland and branch into the gland from the dorsal side.
The sympathetic innervation is managed by the cervical sympathetic trunk and lymphatic drainage passes the paratracheal lymph nodes.(7)

In total thyroidectomies, the gland is removed from the fibrous capsule but carries a risk of injuring the recurrent laryngeal nerve or removing the parathyroid glands. Permanent damage of the recurrent laryngeal nerve occurs in less than 0.4% of operations and can lead to hoarseness if unilaterally, and laryngeal obstruction if bilaterally damaged. The incidence of permanent hypocalcemia due to hypoparathyroidism is less than 1.3%.(8)

2.1.2 Histology

The dominant histological feature of the thyroid gland are the follicles with about 3 million per gland. These follicles vary in size from 50 to 500 µm and are filled with a gelatinous fluid called colloid which is lined with single layered epithelium.
This epithelium consists of follicular cells or thyrocytes that produce thyroglobulin (TG), a glycoprotein that composes the colloid. Thyrocytes form a columnar epithelium during high metabolic activity and flat epithelium when resting.
The small vessels of the thyroid lay between the follicles as do the parafollicular cells. They are also called C-cells, and can be found in small nests of a few cells between the larger follicles although they do not reach into the follicular lumen. They produce calcitonin, which acts to decrease serum calcium levels.(9)

2.1.3 Development

Starting in the third week of gestation, a thyroid placode forms at the endodermal floor of the first pharyngeal pouch. In the following days, it buds out into the root of the tongue and descents down leaving behind the thyroglossal duct. This thyroglossal duct passes through the hyoid bone and usually atrophies, leaving only the foramen caecum at the base of the tongue. Remnants of this duct can form a pyramidal lobe or – rarely - a thyroglossal cyst. The thyroid further descents and forms two distinct lobes. Each lobe then fuses with the ipsilateral ultimobranchial body which derives from the foregut endoderm and develops into the parafollicular cells. The C cells do not originate from the neural crest as previously assumed.(10) First signs of endocrine cells can be seen in the 10th week of gestation with thyroid hormone production starting one week later.(11)

2.1.4 Hormone synthesis and transport

Iodine is an essential chemical element for the body, originally deriving from iodine salts in water. Humans ingest it through their nutrition, derived from animal products such as eggs or dairy, natural water and in higher concentrations from sea products such as marine fish, seafood, or algae. In many landlocked and alpine regions, the nutritional iodine supply cannot be guaranteed and therefore table salt is fortified with potassium iodide by national law since the 1960s. In Austria and Germany, the recommended daily intake for adults is 200 µg but is increased to 230 µg and 260 µg per day for pregnant and lactating women respectively.(12)

The resorption of the ingested iodide occurs in the stomach and small intestine through the *Na^+/I^+-symporter* (NIS).(13) Over 90% of the dietary iodine is absorbed, of which over 90% is then excreted through urine and can be used as a measure of iodine supply.(14) The absorbed iodide is carried to the thyroid gland by the bloodstream, where it is transported by the secondary active NIS in the basolateral membrane of thyrocytes. Due to the strong electrochemical gradient, the transport into the thyrocytes is dependent on an active efflux of Na^+ by the Na^+/K^+-ATPase. This process is referred to as *iodine trapping,* as the iodide is not able to diffuse back into the bloodstream. NIS expression is stimulated by *thyroid stimulating hormone* (TSH), as would be necessary in times of insufficient iodine supply.

The transporter is inhibited by perchlorate, pertechnetate, lithium and thiocyanate with the two former used for diagnostic and therapeutic clinical purposes. The intracellular iodide is then transported into the follicular lumen through an ion channel in the apical membrane of the thyrocytes named "*pendrin"*. The thyrocytes further produce *thyroglobulin* (TG), a glycoprotein rich in tyrosine, which is transported into the lumen via exocytosis and constitutes the colloid.

Thyroid peroxidase (TPO), bound to the apical membrane, oxidizes the iodide with the help of H_2O_2 provided by a NADPH *(nicotinamide adenine dinucleotide phosphate)* oxidase and binds it to the tyrosine residues. This process, called *organification,* creates *iodotyrosines,* which either have one (monoioidotyrosine) or two (diiodotyrosine) iodide atoms. These iodotyrosines are then coupled by TPO to form *triiodothyronine* (T3) or *tetraiodothyronine* (T4 or thyroxine) depending on the number of iodide atoms. This iodinated TG now acts as a thyroid hormone (TH) reservoir. TPO can be therapeutically inhibited by thioamides, such as propylthiouracil, methimazole or carbimazole. A strong inhibition of thyroid function can also be achieved by ingestion of large amounts of iodide (> 100mg or > 500x the recommended daily intake). High intrathyroidal iodine concentrations inhibit organification in a phenomenon termed *Wolff-Chaikoff effect.*(15) After about ten days an *escape phenomenon* occurs due to reduced NIS expression and thyroid hormone levels revert to normal levels.(16)

When TH demand rises, TG is absorbed by thyrocytes through pinocytosis into their lysosomes, where proteases cleave it to T3, T4 and iodotyrosines. The iodotyrosines are deiodinated by an intracellular dehalogenase and the iodide is recycled. Even though TH are lipophilic, they cannot pass the cell membranes passively and are therefore shuttled by transporter proteins, above all MCT8, MCT10 *(monocarboxylate transporter 8 and 10)* and OATP1C1 *(organic anion transporter 1 C1)*.(17)

The thyroid gland releases about 100 µg TH per day, about 90% T4 and only 10% T3. 99.9% of the thyroid hormones in the bloodstream are bound to carrier proteins with only about 0.1% being metabolically active as unbound free T3 (fT3) or free T4 (fT4). About 70% of TH are bound to *thyroxin-binding globulin*, the rest is split up between *thyroxin-binding prealbumin* and albumin. These transport proteins facilitate a long TH half-life of 1 week for T4 and about 1 day for T3.(18-20)

2.1.5 Hormone function

Most of the effects of TH are carried out by T3, as it has a 10x stronger receptor activity, making T4 effectively a prohormone that is mainly important for storage and transport. As most of the excreted hormones are T4, the molecule is transformed into its active form in peripheral tissue by three enzymes called *iodothyronine deiodinases*, namely DI, DII and DIII. These proteins use selenocysteine as a cofactor and can deiodinate either the inner ring or outer ring of the T4 molecule. Outer ring deiodination (by either DI or DII) converts T4 to T3 and therefore activates it, inner ring deiodination (by DI or DIII) converts T4 to reverse T3 (rT3) and deactivates it.(21) Deiodinases can be inhibited by propranolol and cortisol, but also by rT3 itself.(22)

Thyroid hormone action can be classified either by mode of action or target organ system. In the classical *genomic pathway*, TH enter the cytoplasm through specific transporters, thyroid hormone channels, and bind to the cytosolic thyroid hormone receptor.(17) Upon activation this ligand receptor complex relocates into the nucleus and acts as a transcription factor. In the *non-genomic pathway* TH bind to the integrin *αvβ3* on the surface of the target cells and - depending on integrin subtype – initiate different kinases involved in cell growth and proliferation such as ERK 1/2 (*extracellular signal-regulated kinases 1 and 2*) or PI3K (*phosphatidylinositol 3 kinase*).(19, 23-25)

Many thyroid effects have been understood on a molecular level, but not all of them have been thoroughly described.

Fetal development

In the fetus, TH play important roles in the development of several different organ systems: In the brain, they lead to stimulation of axon development, dendrite branching and myelinization, they stimulate growth hormone synthesis and help differentiation of osteo- and chondroblasts, photoreceptors and the inner ear.(26) Therefore, congenital hypothyroidism, if left untreated, manifests as a disorder historically referred to as *cretinism* that encompasses impaired mental development, short stature and deaf mutism.(27)

In Austria, newborn screening for metabolic disorders including congenital hypothyroidism has therefore been introduced in 1966.(28)

Cardiovascular effects

TH act via direct and indirect stimulation of the Ca-ATPase in the sarcoplasmic reticulum and the expression modulation of myosin heavy chain isoforms in cardiomyocytes and myocytes. All these effects lead to increases in speed and force of systolic contraction and diastolic relaxation. TH reduce the expression of angiotensin and stimulate nitric oxide production, which collectively reduces the vascular tone. TH further promote angiogenesis.(29) In myocytes, TH shift the expression towards fast twitch muscle fibers in addition to the earlier mentioned metabolic changes.(23, 30)

Metabolic effects

TH increase the basal metabolic rate and turnover of macromolecules, mainly lipids, through combined catabolic and anabolic effects with a net anabolic effect.

Lipid and cholesterol turnover is stimulated through increased lipolysis, by an elevated expression of low-density lipoprotein (LDL) receptors and by enhanced elimination through bile acids. Here, TH have a net negative effect on lipid and cholesterol levels.(23) In addition, de-novo lipogenesis and fatty acid esterification is also increased.(31)

Glucose metabolism is modulated by TH through stimulation of gluconeogenesis, glycolysis, and intestinal glucose uptake with a net positive effect on glucose levels.(23)

Protein turnover is increased as well. In the skin, this affects the metabolism of glycosaminoglycans such as hyaluronic acid.(32, 33) TH further stimulate sweat and sebaceous glands. In addition to increased protein turnover, TH promote osteoblast and osteoclast proliferation activity in the bone, leading to increased bone remodeling and net loss of bone.(34)

Thermogenesis effects

TH stimulate thermogenesis through central stimulation in the hypothalamus(23) and peripherally in brown adipose tissue through induction of *thermogenin*, which uncouples the respiratory chain in mitochondria and leads to heat generation. They further stimulate brown adipose tissue through stimulation of the sympathetic nervous system and provide free fatty acids as fuel through lipolysis.(31)

Endocrine effects

TH affect the production, response and clearance of numerous other hormones. Under TH stimulation, growth hormone production and response is raised, gonadotropin secretion is increased, as well as cortisol production and clearance are stimulated.(35) TH affect growth hormone and IGF-1 (*insulin-like growth factor 1*) levels in the circulation as well as the pituitary.(36) They have a permissive effect on catecholamines through increased expression of β-adrenergic receptors (37), and the expression of erythropoietin is increased.(38)

2.1.6 Regulation

Blood TH levels are subject to an intricate regulation in the hypothalamus, pituitary gland, and thyroid gland. This (multiloop) feedback system termed hypothalamic-pituitary-thyroid axis is one of the most well-known endocrine systems.

Neuroendocrine cells in the hypothalamus sense declining TH levels and react by secreting *TSH releasing hormone* (TRH) into a portal venous system that imbues the pituitary gland. Here, it binds to the TRH receptor of the anterior pituitary and leads to a release of TSH and prolactin into the blood stream. The TSH receptor, which is mainly found on thyrocytes, is activated, and increases transcription of enzymes required for thyroid hormone synthesis. In addition to increasing the production and release, it also stimulates the uptake of iodine and induces thyrocyte growth. TH and TSH have a log-linear relationship in which even minute changes in TH levels lead to large changes in TSH levels.(39)

In turn, T3 inhibits the expression of TRH receptors and synthesis of TRH and TSH.(2)

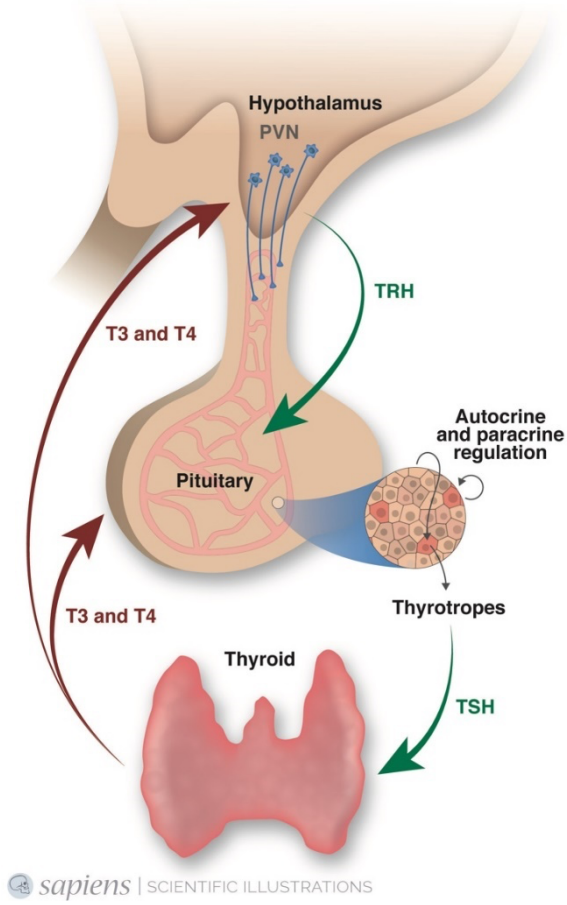


Figure 2 Hypothalamic-pituitary-thyroid axis(2)

Besides the hypothalamic-pituitary-thyroid axis, regulation of TH activity can also be achieved through regulation of NIS expression and therefore iodide intake or deiodinase expression can promote peripheral TH activation. In addition, different hormones can modulate TSH levels directly and indirectly. TSH levels can be reduced by cortisol, dopamine, and somatostatin. Cold stimulus, noradrenaline, and estrogen increase TSH levels with the former being the most potent stimulus for thyroid hormone production.(23)

2.1.7 Hormone metabolism

The main metabolic pathways of TH are deiodination and conjugation in the liver and kidneys. T3 and rT3 can be further deiodinated into T2. These mostly inactive forms (rT3 and T2) are the substrate for sulfonation and glucuronidation that both take place in liver and kidney. These conjugated metabolized forms of TH can then be excreted through bile and urine. Conjugated TH in the intestinal tract can be deconjugated and reabsorbed, thereby acting as a reservoir for TH.(40)

2.2 Autoimmune thyroid disease

2.2.1 Definition

Autoimmune thyroid disease is a group of organ-specific autoimmune disorders directed against the thyroid gland. Due to yet unknown mechanisms, the organ`s self-tolerance is disturbed, which leads to a lymphocytic infiltration of the glandular parenchyma and antibody production against TG, TPO, and TSH receptor. The two main entities in AITD are Hashimoto's thyroiditis (HT) and Grave's disease (GD). These two disorders are grouped together since they share etiology, pathophysiology and sometimes key characteristics of the other disease and sometimes show mutual manifestation.

In HT, the autoimmune process leads to a destruction of the glandular tissue that results in hypothyroidism. As this process takes a lot of time, patients can remain in a state of *subclinical hypothyroidism* (scH) until the compensatory capabilities of the gland are exhausted. In GD, TSH receptor antibodies (TRAb) can activate the receptor and lead to a stimulation of TH release and therefore hyperthyroidism, but there are also inactive TRAbs. Overt hypothyroidism is defined by low T3 and T4 levels in combination with TSH levels above around 4 mU/L, latent or subclinical hypothyroidism is defined by raised TSH levels while peripheral TH levels are normal. Hyperthyroidism shows high T3 and T4 levels with suppressed TSH with levels below around 0.25 mU/L.

2.2.2 Epidemiology

AITDs are the most common autoimmune disorders. HT in particular is the most common AITD with an incidence of 60 – 350/100.000 per year and affects up to 5% of the population.(41) Even though only 20 – 30% of HT patients suffer from hypothyroidism, it is the most common cause of hypothyroidism in iodine-sufficient areas. HT incidence is higher in women, with increasing age and in iodine-sufficient or iodine-replete areas. While the TSH levels continuously increases with age, HT incidence is highest between the third and fifth decade of life. People of European and Asian descent are more likely to develop AITD while people of African descent are less at risk.(4)

Antibodies against thyroid structures can be detected in about 10% of the general population.(42) The worldwide incidence is rising.(43)

2.2.3 Etiology

As with other autoimmune disorders, the definite pathological mechanism is yet to be elucidated. However, two main classes of susceptibility factors can be distinguished:

Genetics

Epidemiologic data suggest that genetics play a major role in AITD, with concordance rates of 0.3 and 0.1 for monozygotic and dizygotic twins respectively.(44) Detectable thyroid antibodies are found in 80% of monozygotic and 40% of dizygotic twins.(45)

In line with the generally higher incidence of autoimmune disorders AITD are also observed at 4 – 10x the rates in women.(4) Hormonal factors seem to play a major role, as is seen in pregnancy where the incidence of HT is relatively low and a spike in HT risk appears in the postpartum period. In addition, women have generally higher levels of antibodies.(46) However, estrogens and androgens are thought to play a somewhat protective role against autoimmune disorders.(46) The drop in estrogen levels would explain some of the increased risk in the postmenopause. Although pregnancies were suspected to be a risk factor due to microchimerism multiparous women generally have the same risk as nulliparous women.(45) Results of the influence of X chromosome inactivation are conflicting.(47, 48)

Polymorphisms associated with higher AITD prevalence have been found for immune modulating genes as well as thyroid-specific genes.(49) The strongest correlation is seen in polymorphisms of the CTLA-4 (*cytotoxic T-lymphocyte-associated protein 4)* and PTPN22 *(protein tyrosine phosphatase non-receptor type 22)*, which both inhibit T cell function.

The vitamin D receptor can be found on many immune cells and polymorphisms, as well as general vitamin D deficiency, are associated with increased AITD risk and increased TSH.(43) TG and TSH receptor polymorphisms are also associated with a slightly higher risk of AITD. TPO polymorphisms, on the other hand, didn't seem to affect susceptibility.(44)

Overall, genetic susceptibility is an important factor, despite the fact that each individual gene has little significance. Furthermore, ethnicity seems to modulate the impact of these genes.(45)

Environmental factors

Iodine

While iodine-deficient areas have a higher incidence of hypothyroidism, the incidence of AITD is lower. Iodine repletion through fortification of table salt for example lead to a significant increase in thyroid antibody prevalence.(4) The underlying mechanism is not yet understood but a higher iodination of TG seems to lead to a higher antigenic potential.(45)

Selenium

This essential element is found in water, seafood, meat, and grains and is necessary for the synthesis of *selenoproteins* that support antioxidant functions, but also iodothyronine deiodinases. Selenium deficiency is associated with a significantly higher risk of AITD.(50)

Nutrition

Western diet leads to increased proliferation and a hyper-responsive state of myeloid cells that respond more aggressively to secondary triggers.(51) Further, BMI shows a positive correlation with TSH and fT3 levels.(52)

Smoking

Smoking leads to a dose-dependent decrease in TSH, increase in thyroid gland size and increased risk of goiter and thyroid nodules. While smoking is an independent risk factor for the development of GD and thyroid ocular disease, it seems to play a somewhat protective role in HT. Furthermore, smoking cessation seems to transiently increase the HT risk.(53)

Medication

Both amiodarone and lithium lead to increases in thyroid antibodies and risk for AITD.(41) Immune checkpoint inhibitors such as ipilimumab or nivolumab cause autoimmune thyroiditis in some patients with a distinct progression of mild thyrotoxicosis that resolves to hypothyroidism.(44) However, they more often lead to GD. Interferon α also shows a significant association with AITD, especially in combination with a chronic hepatitis C infection, as both are independent risk factors.(41)

2.2.4 Pathophysiology

Self-antigens, such as TG, do not elicit an immune response through a mechanism of self-tolerance or *immune tolerance* under physiological conditions. Autoimmune disorders are characterized by a breakdown of this self-tolerance in susceptible individuals and a consequent attack on self-antigens and tissues.

The immune system can be classified into the innate and adaptive immune system. The adaptive immune system is based on a cell-mediated response mainly through T cells and a humoral response through antibodies produced by B cells. Both pathways are mediated by multiple types of *T helper cells* (Th cells) that produce cytokines to support their differentiation into the required cell types. These helper cells express *cluster of differentiation 4* (CD4) on their surface in contrast to *cytotoxic T cells* expressing CD8.

The traditional dichotomy, where a strict distinction between Th1-mediated effects, maintaining cell-mediated immunity, and Th2-mediated effects for humoral immunity has been been made, is no further considered to be unequivocal today as recently cell types such as Th17 or Th22 cells have been discovered.(54)

In AITD, both aspects of the immune response play important roles: Cell-mediated immunity leads to cytotoxic destruction of thyroid tissue and humoral immunity is characterized by production of antibodies against thyroid specific antigens. Data on whether the cellular response precedes the humoral response or vice versa is equivocal. While several risk factors are known, the key mechanism that leads to the immune response is still obscure. One of the suspected mechanisms is the presentation of TG by antigen presenting cells such as dendritic cells to T cells.(55) This triggers the infiltration of the thyroid gland by lymphocytes such as B cells or T cells, both CD4+ and CD8+. These lymphocytes produce cytokines that lead to *major histocompatibility complex* class II production of thyrocytes (through *interferon* γ and *tumor necrosis factor* α by Th1 cells), inducing inflammatory cytokine production by thyrocytes by Th1 cells, and activating apoptosis pathways.(41, 47) Gradually, lymphocytes form secondary follicles in the thyroid tissue with germinal centers(41).

In 1912, Hakaru Hashimoto originally described four hallmark histological features of the *Struma lymphomatosa*(56):

- Diffuse infiltration with lymphoid cells
- Formation of lymphoid follicular centers
- Degeneration and destruction of follicular epithelial cells and colloid
- Increased connective tissue and fibrosis

The lymphocytic infiltrate consists of both B and T cells.(57) As the remaining functional thyrocytes try to compensate the loss in total thyroid function, they undergo oncocytic metaplasia and evolve into large cells with a high amount of mitochondria that is apparent as eosinophilic granula. The cells are then called *Hürthle cells* or *Askanazy cells.*(58)
Besides this cytotoxic attack to the thyroid, there is a humoral attack that consists of IgG *(immunoglobulin G)* autoantibodies against thyroid specific epitopes such as TG, TPO or the TSH receptor, but also NIS or pendrin.(59) TPO-Ab can induce antibody-dependent cellular cytotoxicity by natural killer cells and complement-dependent cytotoxicity.(43) Furthermore, TPO-Ab are associated with lymphocytic infiltration of the thyroid gland and a predictor of thyroid dysfunction. Initially the antibodies are produced in the neighboring lymph nodes, followed by subsequential production in the intrathyroidal lymphoid follicles.(41)
In addition to detectable circulating antibodies, the levels of several cell types and cytokines are altered, such as Tregs, CD8+ cells, IL-17, IL-22. While circulating B cell levels appear to remain unchanged, they even show functional alterations that are assumed to be one of the key events leading to the breakdown of self-tolerance.(45).

Different forms of HT can be distinguished(60):
Classic form: This is the most common variant and incidence culminates around the fifth decade of life, mostly in women. The thyroid gland is enlarged and firm, but most patients are euthyroid. 25% show thyroid dysfunction, mainly hypothyroidism.
Fibrous/atrophic form: This variant becomes increasingly common with age and is again more common in women. Most patients become hypothyroid but may also show goiter. This form shows the strongest association with myxedema.
Juvenile form: This form is defined by an onset before 18 years of age. The thyroid function as well as the progression can be highly variable.

Silent/Painless form: This variant shows a triphasic progression with initial hyperthyroidism due to thyroid cell destruction, then hypothyroidism and in 80% of cases full recovery. Postpartum thyroiditis is defined as a thyroiditis appearing within 12 months postpartum and shows a similar pattern. About 20% of patients with postpartum thyroiditis develop HT especially when TPO-Ab are present.(43)
IgG4 form: This form was only recently discovered and is characterized by its more balanced gender ratio, earlier manifestation, more aggressive development, and higher antibody titers. IgG4 producing plasma cells are detectable in the lympho-plasmocytic infiltrate.(61)

AITD shows a strong association with other autoimmune disorders with as many as 25% of HT patients affected.(62) The most common concomitant disorders are vitiligo, celiac disease, myasthenia gravis, autoimmune gastritis, inflammatory bowel disorders or autoimmune connective tissue disorders.(43) Recent reports on an increased co-occurrence of polycystic ovary syndrome (PCOS) and AITD may be based on immunological phenomena in both entities.(46)
AITD are most common manifestations of autoimmune polyendocrine syndrome type 2, a disorder characterized by the autoimmune affection of multiple endocrine organs. However, only a small fraction of HT patients is affected.(63)

2.2.5 Signs and symptoms

25-30% of HT patients have thyroid dysfunction, ranging from subclinical to overt hypothyroidism. In contrast to many autoimmune disorders including GD, where the autoimmune inflammation itself causes symptoms, this is generally not the case in HT. With the exceptions of goiter formation by inflammatory processes during early stages of the disorder and non-specific rheumatic manifestations(45), most of the symptoms are caused by ensuing hypothyroidism.(58) While the known molecular mechanisms of TH are discussed above, the focus of the following list is on clinical consequences of hypothyroidism:

Cardiovascular
Hypothyroidism can lead to a wide range of symptoms such as bradycardia, diastolic hypertension, and heart failure but also atrioventricular blocks, pericarditis, pericardial

effusion, or cardiomyopathy. Clinical signs can be atrioventricular blocks, bradycardia, prolonged QTc times and low voltage. Furthermore, chronic hypothyroidism increases the risk of atherosclerosis due to hypertension and dyslipidemia.(29)

Gastrointestinal

Decreased intestinal motility can provoke constipation and pseudo-obstruction up to ileus occurrence.(64) Further symptoms may be caused by associated autoimmune disorders such as inflammatory bowel disease, pernicious anemia or celiac disease. A compression of the esophagus caused by goiter may lead to dysphagia. Small intestine bacterial overgrowth is associated to hypothyroidism and may cause abdominal discomfort, flatulence and bloating.(65)

Dermatological

Accumulation of glycosaminoglycans due to low turnover can manifest as a type of non-pitting edema, called myxedema. Predilection sites include the periorbital region, lips, tongue, hands, and feet, but also vocal cords resulting in dysphonia. Furthermore, low protein synthesis leads to brittle nails and hair as well as potential hair loss.(32, 33)

Metabolic and endocrine

Cold intolerance is a common complaint in hypothyroidism and is thought to be a consequence of reduced sympathetic stimulation.(23)

Hypothyroidism is known to lead to reduced lipid metabolism resulting in elevated blood levels of cholesterol, LDL and triglycerides. Furthermore, it leads to a modest increase in body weight.(23)

Increased TRH levels in primary hypothyroidism not only stimulate TSH, but also prolactin secretion and can lead to hyperprolactinemia. This issue can manifest as galactorrhea, sexual dysfunction or menstrual disturbance and lead to infertility and other associated clinical complaints.(66) PCOS may further contribute to fertility issues. Hypothyroidism is associated with insulin resistance and metabolic syndrome.(67)

Hematological

Anemia is a common clinical sign in hypothyroidism, mostly in the form of normochromic normocytic anemia. There is a range of etiological factors, playing a role e.g. reduced

erythropoietin expression, and/or reduced iron, folate and vitamin B12 resorption due to concomitant diseases or myxedema.(68)

Neuropsychiatric

The clinical presentation of hypothyroidism is unspecific and includes central manifestations such as impaired cognitive function, reversible dementia, depression, ataxia, but also peripheral manifestations such as neuropathies or nerve entrapment syndromes like carpal tunnel syndrome.(66)

One very rare disorder linked with HT is called "*Hashimoto's encephalitis"*. It shows a heterogeneous clinical presentation including altered mental state, seizures, stroke-like episodes or myoclonus and an association to high TPO-Ab titers. The degree of association between the two diseases is insecure, since it seems to be independent from thyroid function and T4 replacement shows no benefit, while glucocorticoid therapy is effective.(69)

Thyroid ocular disease

A small percentage of HT patients have detectable TRAbs and like in GD, this can lead to an inflammation of the retroorbital tissue with consecutive exophthalmos. This can be seen as a case where the definitions of HT and GD are blurred as the symptoms can overlap.

HT patients with ocular disease had TRAb in 70% of cases as opposed to HT patients without ocular disease who only had TRAb in 6% of cases.(44)

Diagnosis and therapy are based on clinical presentation and ophthalmological findings.

2.2.6 Diagnosis

Due to the highly unspecific nature of symptoms, clinicians depend on further biochemical and radiological tests to confirm or rule out the diagnosis.

Biochemistry

TSH, fT3 and fT4 are used to assess thyroid function, hence their name "thyroid function tests". Although TSH is only an indirect indication of thyroid function, it is the most sensitive due to its log-linear relationship to TH and therefore the first line analyte. TSH alone cannot differentiate between subclinical and overt hypothyroidism and the reference values increase with age, from 4 mU/L in young adults up to 7 mU/L in geriatric patients.(67) Some authors

even refer to mild scH in higher age as protective against certain disorders, such as dementia.(66)

To assess TH levels, free T4 levels are mostly used. Free T3 is less commonly used as it only dips in states of severe hypothyroidism. Total TH values are highly dependent on transporter protein levels, which can be variable.(27)

The current reference ranges of the Endocrinology Lab Platform in Graz are:(70)

Free T3 3.0 – 6.3 pmol/L

Free T4 9.5 - 24 pmol/L

TSH 0.1 – 4.0 mU/L

Specific antibodies

In AITD, antibodies against multiple structures may be found, but only three are widely used in diagnostics. These are antibodies directed against TG, TPO and the TSH receptor, TG-Ab, TPO-Ab and TRAb, respectively. TPO-Ab can be detected in 95% of HT patients at the time of diagnosis, TG-Ab in about 70% and TRAb in about 10%. The supposed explanation is based on the chronology of the disease. TG-Ab seem to play a larger role in the early stages of the autoimmune process, while TPO-Ab are more prominent in later stages, which coincides with the time of presentation.(71) About 10% of HT patients also have detectable TRAbs as opposed to almost 100% of GD patients. They are mostly blocking antibodies, which cannot be detected using normal assays and require more complex assays.(41) TSH receptor stimulating antibodies, however, are associated with thyroid ocular disease symptoms.(44)

The current reference ranges for the Endocrinology Lab Platform in Graz are:(70)

TG-Ab 0 - 60 U/mL

TPO-Ab 0 – 60 U/mL

TRAb 0 – 15 U/L

Ultrasound features

A healthy thyroid gland is hyperechoic, relative to the surrounding cervical musculature and has homogenous parenchyma.

At initial stages, HT can lead to hypoechoic enlargement of the gland or a normally dimensioned isoechoic, but also appear inhomogeneous due to hypoechoic lymphocytic infiltrates. Progressively, a hypoechoic atrophic gland with reduced vascularization in Doppler sonography can be detected. The primary atrophic form is isoechogenic and

homogenous.(72) Anechoic nodules are most likely cysts. Hypo- or hyperechoic nodules are suspicious for a malignancy with certain additional criteria.(73)

Other

Thyroid nodules are a common finding in HT. Even though scintigrams have lost their diagnostic foothold in the advent of thyroid sonography they prove to be useful in differentiating suspicious thyroid lesions. HT shows uniformly reduced uptake in the entire gland, cysts and carcinomas appear as cold nodes. Suspicious lesions warrant a fine needle biopsy to rule out malignancy.(74)

In summary, the diagnosis of HT can be made in patients with presence of TPO- and/or TG-Ab and hypothyroidism. Here, a thyroid sonogram is not required, but can be used to confirm the diagnosis. Absence of antibodies does not rule out AITD. In suspected HT cases with hypothyroidism and negative thyroid antibodies or vice versa, a sonogram should be used to confirm the diagnosis.

2.2.7 Therapy options

In line with other autoimmune disorders, there is no curative treatment for HT. However, in contrast to other autoimmune disorders, immunosuppressive therapy is not particularly effective and causes a variety of side effects that are thought to outweigh any possible therapeutic gains.(45) In addition, glucocorticoids indirectly decrease TSH levels, making TSH monitoring less reliable.(23)

Instead, treatment is based on the replacement of TH in the form of *levothyroxine* (LT4), a synthetic sodium salt of thyroxine. LT4 has a high oral bioavailability of 70 – 80%.(27, 75) The goals of replacement therapy are alleviating the symptomatic burden and avoiding overtreatment.(75) The reduction of the risk of long-term health effects such as *cardiovascular disease* (CVD) or cognitive decline should also be achieved.

TSH monitors correct dosing and was established as a sensitive tool, as it reacts very clearly to minute variations in TH levels. Therefore, TSH acts as a surrogate parameter for thyroid function. The target TSH interval lies between 0.4 – 2.5 mU/L but may be shifted up to 1.0 – 5.0 mU/L in elderly patients, where higher TSH levels seem to play a protective role.

Undertreatment may lead to persistent symptoms and maintains the elevated risk of CVD and neurological disorders. Overtreatment and consequent hyperthyroidism may lead to osteoporosis, atrial fibrillation and a subsequently elevated stroke risk.(75)
Indications for treatment initiation are manifest hypothyroidism and, under certain circumstances, also subclinical hypothyroidism. The rationale behind LT4 treatment in scH was the increased risk of CVD and cognitive deficits and could therefore be seen as a preventive measure rather than therapy as scH rarely leads to symptoms. However, the association with increased risk of atrial fibrillation, heart failure, stroke, coronary heart disease and overall increased mortality in scH could not be confirmed. ScH has a conversion rate to manifest hypothyroidism of 2% per year in TPO-Ab-negative and 4% per year in TPO-Ab-positive patients.(76) Over 50% of patients with mild scH (TSH < 10 mU/L) revert back into euthyroidism in 5 years. LT4 treatment does not lower the risk of conversion or long-term health risks or improve quality of life in scH.(77) Therefore, a broad recommendation of LT4 therapy in scH is being questioned recently and the few remaining indications are TSH > 20 mU/L, patient age < 30 years, unplanned pregnancy, infertility, severe symptoms of hypothyroidism or patients already on LT4 treatment. Instead, a reevaluation is recommended every 6 – 24 months, depending on the individual risk.

If the decision is made to initiate T4 therapy in manifest hypothyroidism, treatment should start with the full LT4 dose of 1.6 µg/kg body weight. In patients with CVD dose titration up to the full dose is recommended. A LT4 steady state is reached after 6 weeks therefore follow up assessments should be scheduled after 6 – 8 weeks.(75) The LT4 dose is titrated until the TSH reaches the target interval and the symptoms are alleviated. Patients should be reassessed with a TSH measurement in intervals of 4 – 6 months for the first year and yearly after that.(75)

Additional treatment modalities

Liothyronine

Liothyronine is the synthetic preparation of T3. The rationale behind T3 substitution is bypassing the deiodination route and normalizing TH levels which are skewed towards T4 in LT4 monotherapy. Such as T3, liothyronine has a short half-life and requires 2 – 3 doses per day. This makes it hard to maintain for patients and the fluctuating TH levels make surveillance with TSH cumbersome.

Combination therapy with liothyronine and levothyroxine is a more promising treatment. While most trials don't support its' superiority over LT4 monotherapy, two trials reported improvements in neurocognitive function.(78, 79) A minority of patients under LT4 monotherapy has residual symptoms. While there have been no clinical trials investigating the effects of combination therapy in this group, it is possible that they benefit the most.(80)

Iodine

Iodine intake beyond the minimal recommended intake of around 200 µg is not suggested. Additional substitution doesn't improve thyroid function and can lead to adverse effects.(75)

Selenium

Selenium deficiency is associated with an increased AITD risk. Indeed, selenium supplementation in selenium deficient AITD patients lowers the levels of TPO-Ab and different inflammatory cytokines. The effect is especially pronounced in pregnant women, where the risk of postpartum thyroiditis and rate of conversion into permanent hypothyroidism is significantly lowered. However, a systematic review and meta-analysis showed no effect on disease remission, progression, lowered LT4 dose or improved quality of life. In addition, high selenium status is linked with insulin resistance and type 2 diabetes mellitus. Therefore selenium supplementation should be reserved for selenium deficient individuals.(50)

Vitamin D

Vitamin D deficiency is linked with an increased risk of AITD.(46) Restoration of vitamin D levels appears to decrease thyroid antibody levels in specific patient groups.(81) Similar to selenium, vitamin D supplementation is recommended to deficient individuals.

Surgery

Thyroidectomy is rarely indicated in HT. Patients with suspicious nodules who refuse fine needle aspiration should undergo thyroid surgery to remove the gland along with the lesion for histological examination.(74) Although HT patients are more likely to develop complications after thyroid surgery (82), some patients with persistent symptoms seem to benefit from surgery. General health and fatigue show improvements after total thyroidectomy which may hint at the possibility that TPO-Ab and the immune process itself cause part of the residual symptoms.(83)

2.2.8 Complications

Myxedema coma:

This rare clinical entity with a high mortality of 30% is most probably caused by allostatic stress such as infection, trauma, or surgical intervention on top of severe hypothyroidism. The clinical presentation differs from hypothyroidism in regards of its manifestation, rather than in its quality. The key clinical signs are hypothermia, altered mental status, generalized edema and most significantly, probable cardiopulmonary pathologies, arrhythmias, hypotonia, hypoventilation. Upon admission to the intensive care unit, patients should receive high dose intravenous T4 up to 600 µg and corticoids and should be treated for cardiopulmonary stabilization.(74)

Thyroid malignoma:

Two types of cancer show an association with HT. Primary thyroid lymphoma develops as a consequence of the chronic inflammatory process. HT increases the risk 40- to 80-fold, but due to its low baseline incidence less than 1% of HT patients develop this type of non-Hodgkin lymphoma. Diagnosis is based on sonography, and prognosis is favorable in most cases.(84) Papillary thyroid cancer is assumed to be caused by chronically elevated TSH levels although the evidence for an increased risk in HT is equivocal.(45) Thyroid sonograms should be repeated in intervals of 3 – 5 years.(62) Suspicious lesions should receive a cytological analysis through fine needle aspiration.

2.3 MicroRNAs

2.3.1 Biogenesis

Synthesis of miRNAs starts in the nucleus where miRNA genes are transcribed by RNA polymerase II for the most part.(85) The introns or exons of noncoding primary transcripts of miRNA genes are the basis for most canonical miRNAs, although intergenic regions as well as exons of mRNA precursors are possible sources. The latter are under the same transcriptional regulation as the mRNA.

The transcription product is called a *primary miRNA* (pri-miRNA) and varies in length from 200 to 3000 nucleotides. In the canonical pathway this pri-miRNA consists of multiple short hair pin structures, connected by single stranded RNA and carries a 5'-cap and poly(A) tail like *messenger RNA* (mRNA). Still within the nucleus, the pri-miRNA reaches the *microprocessor complex,* which consists of the ribonuclease III, *Drosha,* and DGCR8 *(DiGeorge syndrome critical region 8)*. Drosha cleaves the pri-miRNA at the base of the hairpin creating the *precursor miRNA* (pre-miRNA). This double stranded pre-miRNA has a length of about 70 nucleotides and still features the distinctive hairpin. The pre-miRNA is channeled through the nuclear membrane into the cytoplasm by *exportin-5* and Ran-GTP *(GTP-binding nuclear protein Ran)*. Here, another ribonuclease III enzyme, *Dicer*, cleaves the pre-miRNA removing the hairpin structure. The resulting stem region is still double stranded and has a length of about 22 nucleotides. An *Argonaute protein* (Ago) unwinds and separates this miRNA-duplex into the mature single stranded miRNA.(86)

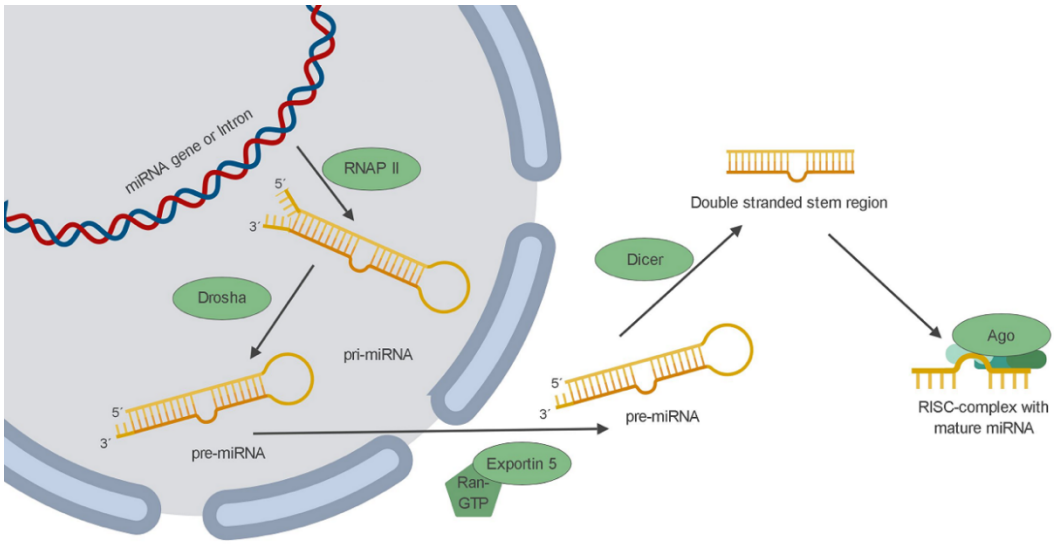


Figure 3 MicroRNA biogenesis adapted from Foessl et al*(87)*

2.3.2 Nomenclature

Mature miRNAs are numbered consecutively in order of discovery independently of species. In animal miRNAs the number is preceded by a dash symbol.
The prefix is dependent on organism, human miRNAs have the prefix *hsa* for homo sapiens. The genes are named after the corresponding miRNA. As the same miRNA can be transcribed from different genes, the different genomic loci are numbered. Related miRNAs share most of their sequence and have the same number but possess an additional a letter as an suffix.
The strand that is read by RISC is the *guide strand* and keeps the miRNA name. The *passenger strand* is added an asterisk. Without this indication, the 5p-strand is the assumed guide strand. The suffixes *-3p* and *-5p* indicate whether the mature miRNA was generated from the 3' or 5' arm of the hairpin precursor.(88)

2.3.3 Function

After DNA is transcribed into precursor mRNA the introns are spliced and the molecule is further modified. This spliced mRNA contains *untranslated regions* (UTR) on each end. A *5' cap* is attached to the 5' UTR and a *poly(A) tail* to the 3' UTR. These steps are called *capping* and *polyadenylation,* respectively, and both increase the stability of the mature mRNA.
For the function of *RNA interference* (RNAi) miRNAs rely on the on the *RNA-induced silencing complex* (RISC). RISC is a ribonucleoprotein complex containing an Argonaut protein, which binds to the miRNA's sugar-phosphate backbone leaving the nucleobase free to interact with target mRNA. However, only a seven-nucleotide long segment ranging from nucleotide 2 to 8, the *seed,* binds to the target mRNA. Based on this seed region miRNAs are grouped into families. The pairing between miRNA and target mRNA happens on the 3' UTR of the mRNA, and depending on the complementarity, the RISC can perform different actions:
In cases of near perfect complementarity, a catalytically active Ago, a *slicer*, cleaves the mRNA at the site of miRNA binding. This *Argonaut-catalyzed mRNA cleavage* is most prevalent in plants and rarely occurs in animal cells.(86)

With incomplete base pairing, Ago in tandem with TNRC6 *(trinucleotide repeat-containing gene 6A protein)* shortens the poly(A) tail and further hinders translation initiation. This *deadenylation* or *translatory repression* accounts for the vast majority of RNAi in animals. Since an intact poly(A) tail is required for translation, this blocks further protein biosynthesis. Further, a shortened poly(A) tail leads to *mRNA decapping*, which ultimately results in targeting the mRNA for exoribonucleases.(86)

In addition to these main pathways, miRNAs are also capable of silencing on the transcriptional level and increasing translation.(89, 90)

MiRNAs can either target one specific or multiple mRNAs. Vice versa, mRNAs can be targeted by one or multiple different miRNAs.(91) This redundancy complicates the study of miRNAs as the simple loss of function of one miRNA presumably has little significance. The most effective translational repression occurs through a combination of different RISCs as one miRNA can only reduce translation by about 20%.(86)

MiRNAs generally play a role in the development of virtually every organ system.

They modulate various physiological processes such as lipid and glucose metabolism, blood pressure or bone resorption but can also involve malignant transformation.(86, 92)

2.3.4 Transport

Although initially believed to be solely intracellular molecules, miRNAs were soon detected in various different body fluids, first and foremost blood, but also cerebrospinal fluid, saliva or urine.(93)

While the extracellular space is a very hostile place for RNAs due to an abundance of RNAses, miRNAs stand out by being remarkably stable. They are even more stable than the mRNAs they interact with and survive freezing, thawing cycles, but also different pH values.(94) Different transport vehicles facilitate their survival and transport into distant tissues. The two pathways are extracellular vesicles and proteins.

The main types of vesicles are exosomes and microvesicles, which differ in size and biogenesis. While microvesicles are larger and created by direct outward budding, exosomes require multiple steps of inward and outward budding.

However, most miRNAs are transported via proteins, especially *ribonucleoproteins* such as Ago. They can also be carried by *high density lipoproteins* (HDL), LDL or *ribonucleophosmin 1*.

Vesicle loading and uptake into target cells have been subject to ongoing research providing evidence suggesting highly specific mechanisms. Due to varying miRNA concentrations between parent cell and transport vehicle a specific loading process is suspected. While extracellular vesicles may fusion with the plasma membrane and release their contents into the cytoplasm, active uptake is necessary for protein bound miRNAs.

In the circulation they can act as endocrine mediators and facilitate fetal-maternal crosstalk and tumor metastasis.(92, 95)

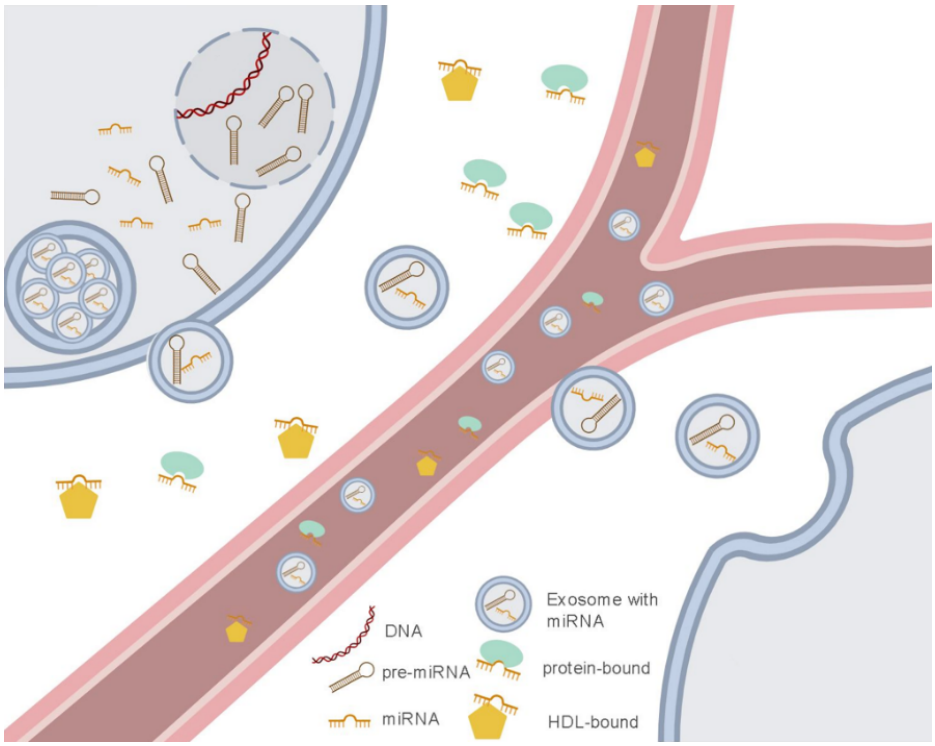


Figure 4 MicroRNA transport adapted from Foessl et al.*(87)*

2.3.5 MiRNAs in autoimmunity

MiRNA genes constitute 1% of the genome and are estimated to target 30-50% of human mRNAs.(86, 96) Because of the wide array of interactions on virtually every facet of the metabolism, it has long been speculated that they may play a role in the immune system.

Indeed, miRNAs have been shown to modulate to numerous immunological pathways through various mechanisms and are themselves subject to regulation through cytokines. MiRNAs have been linked to different stages of lymphocyte development and differentiation and the function of different cell types, such as T cell, B cells, macrophages, and dendritic cells. Immune responses can be stimulated or inhibited depending on the specific miRNA.

Sometimes the same miRNA can have opposing effects in different underlying diseases.(91, 97) The following examples are intended to illustrate some of the most studied relationships:

MiR-let7 interferes with many steps of T cell metabolism, modulates the expression of different cytokines, such as *interleukin 6* (IL-6), IL-10, IL-17 or TNF-α *(tumor necrosis factor α)*.(98, 99)
MiR-146a is regarded as one of the key regulators of T cell response and is able to broadly inhibit the innate immune system.(99) It negatively regulates the differentiation of Th1 and Th2 cells through interference with interferon signaling.(91, 97, 99)
MiR-155 indirectly maintains immune responses by stimulating the production of TNF-α, IL-6 and IL-17. On the other hand, it also augments Treg function through increased FoxP3 *(forkhead box P3)* expression, the main regulator of Treg function. This dampens Th1 differentiation.(91, 97)
In addition to these effects miRNAs can directly bind to specific receptors such as *Toll like receptors*.(99) Differential expression of these and many other miRNAs has been observed in various autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis.(91)

2.3.6 miRNAs in AITD

Differentially expressed miRNAs in AITD have been detected in thyroid tissue and blood.
The majority of studies investigate their role as diagnostic and prognostic parameters. The most investigated miRNAs are miR-Let7, -21, -142, -146, -155, 375 and -451.(95, 100, 101)
Several miRNAs show correlations with antibody levels, such as miR-Let7, -21, -96, -125, -142.(102, 103)
Interestingly MiR-142-5p inhibits the expression of *claudin*, a protein important for tight junctions between thyrocytes, an important feature that blocks the immunogenic colloid from lymphocytes.(103)

3 Material and methods

3.1 Preparation

3.1.1 Literature review

A literature search was performed using PubMed (United States National Library of Medicine). Articles in English and German language were used, irrespective of country of origin. Original articles and reviews published mainly between 2000 and 2018 represent the main sources. Pediatric and geriatric studies were excluded, as were case reports. Reference lists of the articles and textbooks were examined for further information. The phrases and key words were “AITD”, “Hashimoto” “miRNA”.

3.1.2 Study design

The choice of miRNAs was largely based on the 2018 paper by Martinez-Hernandez et al. and Yamada et al.(100, 101) The investigated miRNAs are listed in Table 1. The analyzed miRNA signature corresponds with the miRNA signature used by Trummer et al.(3) and is listed in Table 1.

The core question was:
Do these specific miRNAs differ between patients with AITD and healthy controls in our study cohort?
The alternative hypothesis assumes a difference between the studied groups, the null hypothesis assumes the miRNAs do not differ significantly.

The secondary question was:
Are miRNAs useful for determining prognosis in HT?
The alternative hypothesis assumes a difference in clinical parameters after one year in participants with differentially expressed miRNAs, the null hypothesis assumes no such correlation.

The study type was a monocentric observational study. Due to the high incidence and prevalence of AITD, a prospective approach was chosen as recruitment of sufficient numbers of patients are possible within the framework of a diploma thesis.

microRNA	**Mature sequence** ***(3)***
miR-21-5p	5′ UAGCUUAUCAGACUGAUGUUGA
miR-22-3p	5′ AAGCUGCCAGUUGAAGAACUGU
miR-22-5p	5′ AGUUCUUCAGUGGCAAGCUUUA
miR-96-5p	5′ UUUGGCACUAGCACAUUUUUGCU
miR-142-3p	5′ UGUAGUGUUUCCUACUUUAUGGA
miR-146a-5p	5′ UGAGAACUGAAUUCCAUGGGUU
miR-301-3p	5′ CAGUGCAAUAGUAUUGUCAAAGC
miR-375	5′ UUUGUUCGUUCGGCUCGCGUGA
miR-451	5′ AAACCGUUACCAUUACUGAGUU

Table 1 Mature microRNA sequences

3.1.3 Ethical committee submission

A study protocol was created, where the main points of the study such as core question, type of study, estimated sample size were recorded. The sample size was calculated with the help of a trained statistician. A questionnaire was created for the most common symptoms. The anthropometric data and past medical history were recorded on a source data form.
Informed consent waivers for participation in an observational study, genetic study and for biobanking were composed.
A proposal with the supplemental documents above was submitted to the Ethics Committee of the Medical University of Graz. Approval was obtained after an amendment regarding the recruitment of healthy control participants. The study was conducted in accordance with the principles of the Declaration of Helsinki.

3.2 Data collection

3.2.1 Study recruitment

Patients were recruited at the Department of Endocrinology and Diabetology's outpatient clinics by doctors who identified patients and through posters with contact information distributed throughout the Department of Internal Medicine and the Library of the Medical University of Graz.

The inclusion criteria were age between 18 to 70 and signed informed consent waivers. Patients were excluded if they were beyond the age limits, pregnant, in the presence of severe multimorbidity (CVD, metabolic) or non-AITD thyroid disease (congenital hypothyroidism, thyroid cancer) and if the informed consent waivers were not signed.

Recruitment started in May 2019 and had to be stopped prematurely in March 2020 as a direct consequence of the Covid-19 pandemic. Due to this termination, the collected data only allowed for a cross-sectional analysis as no follow up assessments were possible.

3.2.2 Assessment

After contacting the study coordinator, the patients were assigned an appointment for the patient visit. The visits were scheduled for the morning after an overnight fast to account for circadian changes and prandial influences.

During the appointment, patients received written informed consent waivers regarding general information about the study, genetic analysis, and preservation of blood (*biobanking*). In addition, they were personally informed by trained physicians. Here, the first part of the source data form was filled out by physician, where notes of the patient history, such as medication, were taken. Then, the patients went through several stations:

Questionnaire

First, patients were asked to fill out the questionnaire to screen for the typical and most common symptoms of thyroid dysfunction including altered appetite, diarrhea/constipation, heat/cold intolerance, tremor, insomnia, fatigue, muscle weakness, bradycardia/tachycardia, edema, dry skin, hoarseness, hair loss, weight change, menstrual disorders.

Imaging

Thyroid sonography was performed by trained endocrinologists. Measurements of the gland's length, width and depth were taken and the total thyroid lobe volume was measured through L x W x D x 0,5.(104) The values of each side were calculated to obtain the total thyroid gland volume. Further morphological features such as echogenicity, nodules and circulatory changes were recorded.

Bone density was measured via dual-energy X-ray absorptiometry and high-resolution peripheral quantitative computed tomography in some patients but could not be applied due to technical difficulties.

Anthropometric data

Body height, weight as well as waist and hip circumferences were measured by trained nurses. These measurements were used to calculate the BMI (body mass index) and hip-to-waist ratio.

Laboratory analysis

33 mL of blood were drawn by a trained nurse. 3 mL each of EDTA blood were used for a complete blood count, genetic analysis and endocrinological analysis of bone metabolism.

8 mL of serum were used for endocrinological parameters and miRNA analysis.

8 mL of lithium heparin blood were used for clinical chemistry and immunological analysis.

8 mL of urine were used to analyze iodine excretion.

Data from the general laboratory, as well as endocrinological and immunological laboratories were accessed digitally. Data from the SDFs, questionnaires and sonography data were recorded manually. Data from the questionnaires, imaging and most of the laboratory analysis was originally intended to be used for prognostic information.

3.2.3 Laboratory methods

The EDTA samples were used for a complete blood count and were analyzed using an automated hemogram via simple flow cytometry. Biochemical parameters such as electrolyte levels and liver and kidney function tests were analyzed using mainly photometry, but also potentiometry, immunonephelometry, immunoturbidimetry or

electrochemoluminescent immunoassays for certain parameters. The analyses were performed at the Clinical Institute of Medical and Chemical Laboratory Diagnostics.
Immunological parameters such as cytokines were measured using Luminex assays at the immunological laboratory of the Division of Rheumatology and Immunology.
Urinary iodine as an indicator of iodine supply was measured through ash content determination at the Division of Nuclear Medicine of the Department of Radiology.
Endocrinological parameters including free TH levels and thyroid antibodies were analyzed using luminescence immunoassays at the Endocrinology Lab Platform of the Division of Endocrinology and Diabetology.

MiRNA analysis

MiRNA was isolated using the miRNeasy Serum/Plasma Advanced Kit according to the manufacturer's instructions. After elution with RNAse-free water and centrifugation the isolated miRNAs were frozen and stored at −80 °C. *Complementary DNA* (cDNA) was generated using miRCURY LNA RT synthesis kit and subsequent *quantitative real-time polymerase chain reaction* (qRT-PCR) was performed in duplicates using miRCURY LNA SYBR Green PCR Kit and specific miRCURY LNA miRNA PCR Assays (all kits and assays from Qiagen, Hilden, Germany) with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).
To control the quality and efficiency of RNA isolation, cDNA synthesis and PCR amplification exogenous oligonucleotides were used (RNA Spike-in Kit for RT, Qiagen, Hilden, Germany). Spike-in controls (UniSp2, UniSp4, UniSp5) in known concentrations are added prior to the RNA isolations and cDNA synthesis. The concentrations decrease in 100-fold decrements from UniSp2 to UniSp4 and again from UniSp4 to UniSp5 with UniSp5 corresponding with the miRNAs with the weakest expression. A fourth spike-in, UniSp6, and synthetic cel-miR-39-3p are added to control for cDNA synthesis.

3.3 *Data preparation*

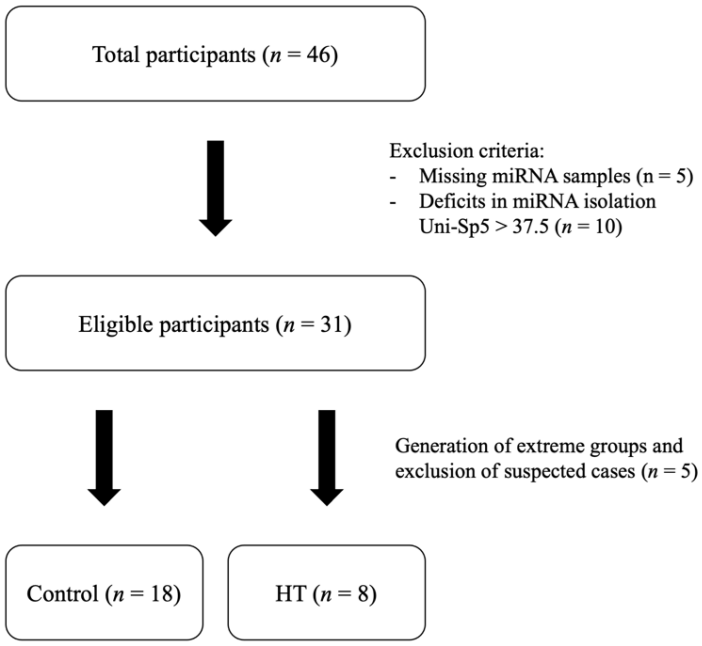


Figure 5 Study population

To account for fluctuations in miRNA extraction and amplification, interplate calibration is applied. Up to 40 cycles were performed in duplicates to calculate average *cycle threshold* (Cq) values. Samples with Cq ≥ 37.5 were excluded from further analysis. ΔCq values were calculated by normalizing the average Cq values to the exogenous spike-ins. ΔΔCq values were calculated by subtracting ΔCq values of healthy controls from ΔCq values of HT patients. Ultimately fold change can then be calculated as $2^{-\Delta\Delta Cq}$.(105)

3.4 Statistical analysis

Data analysis was performed with IBM SPSS Statistics version 28. (IBM, Armonk, New York) Quantitative data were represented as means ± standard deviation, qualitative data were represented as a percentage. To test the ΔΔCq values for normal distribution the Kolgomorov-Smirnoff test was used, and the quantile-quantile plots were also nvisually evaluated. Variance homogeneity was determined using the Levene test. Parametric testing using the student's *t*-test was performed on normally distributed quantitative date. Non-parametric testing using the Mann-Whitney *U* test was applied to unequally distributed data. The requirements for the *t*-test were fulfilled in the group with ΔΔCq. The *p*-values were two sided and the statistical significance of 0.05 was chosen. The resulting data were visualized using GraphPad Prism version 5. (GraphPad Software Inc., California, US)

4 Results

4.1 Study population

Out of the total 46 participants 15 had to be excluded due to missing miRNA samples or insufficient RNA isolation efficiency. 5 participants showed inconclusive results suspicious of developing HT. The method of extreme groups was used to generate more homogenous groups and the third group of subjects was then excluded from further statistical analysis.
A total of 26 participants, 88% women and 12% men, were included in the analysis. Of these, 8 were previously diagnosed with HT, all being female. 18 participants were classified as healthy, 83% women and 17% men, with TSH values in the normal range, undetectable thyroid antibodies with no pathological ultrasound findings. All participants were non-smokers. Further data is noted in table 2. In our study cohort there was no significant difference in age or thyroid parameters between both groups. TG-Ab values below 20 U/mL and TPO-Ab values below 10 U/mL were not detectable and were calculated as 20 U/mL and as 10 U/mL respectively. TG-Abs were detectable in no participants in the healthy group and 4 participants in the HT group. TPO-Abs were detectable in 8 participants in the healthy group and 5 participants in the HT group.

	Control group	**HT group**
n	18	8
Gender (F/M)	15/3	8/0
Age (years)	42.9 ± 15.6	43.3 ± 13.0
fT3 (ng/dL)	5.2 ± 0.6	5.0 ± 0.4
fT4 (ng/dL)	14.5 ± 1.6	15.5 ± 3.7
TSH (µU/mL)	2.2 ± 1.3	2.2 ± 1.2
TG-Ab (U/mL)	< 20	420.0 ± 1043.7
TPO-Ab (U/mL)	13.3 ± 5.3	238.9 ± 340.2

Table 2 Comparison between two groups control and HT

4.2 MiRNA expression

It is important to keep in mind that due to the negative logarithmic relationship between ΔΔ*Cq* and *fold change,* negative ΔΔCq values represent higher expression and higher ΔΔCq values represent lower expression of the miRNA.

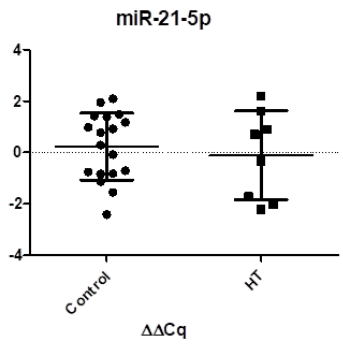


Figure 6 Scatter plot miR-21-5p comparison between controls and HT patients

The mean ΔΔCq values of miR-21-5p are 0.23 ± 1.31 for healthy controls and -0.10 ± 1.72 for HT patients. The *p*-values in parametric and non-parametric testing are 0.587 and 0.617, respectively.

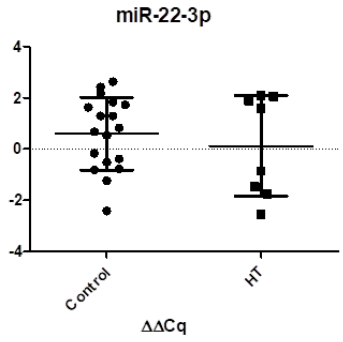


Figure 7 Scatter plot miR-22-3p comparison between controls and HT patients

The mean ΔΔCq values of miR-22-3p are 0.60 ± 1.41 for healthy controls and 0.13 ± 1.97 for HT patients. The *p*-values in parametric and non-parametric testing are 0.493 and 0.541, respectively.

The HT group appears to consist of two distinct subgroups with either low or high levels of miR-22-3p. In patients with high levels of circulating miR-22-3p the TSH level was 1.4 ± 1.1, in patients with low levels of miR-22-3p 3.0 ± 0.9. Subgroup data is noted in table 3. Due to the small sample size no further statistical analysis was possible.

	low ΔΔCq-miR-22-3p	high ΔΔCq-miR-22-3p
n	4	4
Age (years)	42.0 ± 12.4	44.5 ± 15.4
fT3 (ng/dL)	5.1 ± 0.4	4.9 ± 0.5
fT4 (ng/dL)	16.7 ± 5.1	14.3 ± 1.4
TSH (µU/mL)	1.4 ± 1.1	2.9 ± 0.8
TG-Ab (U/mL)	42.0 ± 44.0	798.0 ± 1469.3
TPO-Ab (U/mL)	165.3 ± 206.5	312.5 ± 461.4
LT4 treatment	3 (75%)	2 (50%)

Table 3 miR-22-3p subgroups

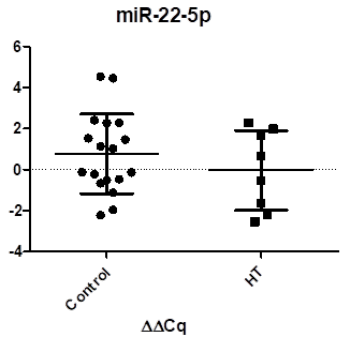


Figure 8 Scatter plot miR-22-5p comparison between controls and HT patients

The mean ΔΔCq values of miR-22-5p are 0.76 ± 1.97 for healthy controls and -0.04 ± 1.96 for HT patients. The *p*-values in parametric and non-parametric testing are 0.342 and 0.345, respectively.

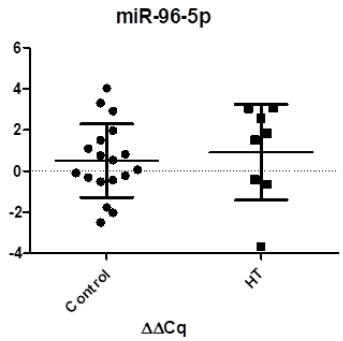
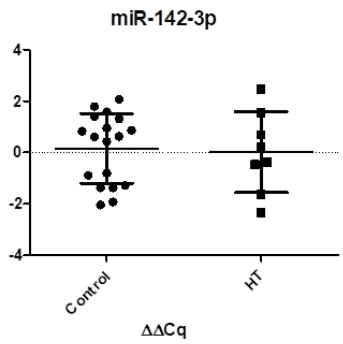


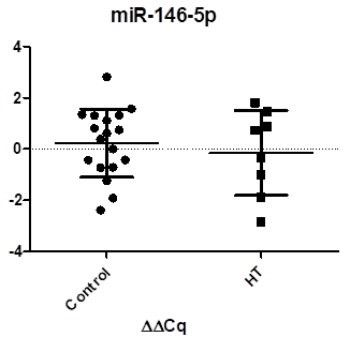
Figure 9 Scatter plot miR-96-5p comparison between controls and HT patients

The mean ΔΔCq values of miR-96-5p are 0.51 ± 1.78 for healthy controls and 0.91 ± 2.34 for HT patients. The *p*-values in parametric and non-parametric testing are 0.630 and 0.541, respectively.



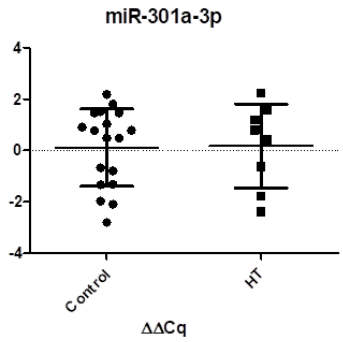
The mean ΔΔCq values of miR-142-3p are 0.16 ± 1.36 for healthy controls and 0.01 ± 1.58 for HT patients. The *p*-values in parametric and non-parametric testing are 0.816 and 0.739, respectively.

Figure 10 Scatter plot miR-142-3*p* comparison between controls and HT patient



The mean ΔΔCq values of miR-146-5p are 0.24 ± 1.33for healthy controls and -0.15 ± 1.66 for HT patients. The *p*-values in parametric and non-parametric testing are 0.532 and 0.781, respectively.

Figure 11 Scatter plot miR-146-5p comparison between controls and HT patients



The mean ΔΔCq values of miR-301a-3p are 0.11 ± 1.51 for healthy controls and 0.18 ± 1.63 for HT patients. The *p*-values in parametric and non-parametric testing are 0.912 and 0.824, respectively.

Figure 12 Scatter plot miR-301a-3p comparison between controls and HT patients

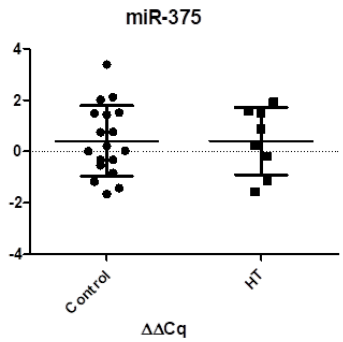


Figure 13 Scatter plot miR-375 comparison between controls and HT patients

The mean ΔΔCq values of miR-375 are 0,42 ± 1.37 for healthy controls and 0.41 ± 1.30 for HT patients. The *p*-values in parametric and non-parametric testing are 0.985 and 0.781, respectively.

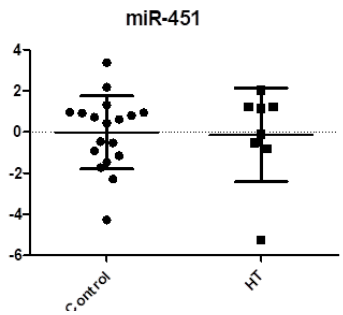


Figure 14 Scatter plot miR-451 comparison between controls and HT patients

The mean ΔΔCq values of miR-451 are -0,03 ± 1,77 for healthy controls and -0.14 ± 2.29 for HT patients. The *p*-values in parametric and non-parametric testing are 0.899 and 0.579, respectively.

In conclusion, there was no significant difference between healthy controls and HT patients in the expression of the nine analyzed miRNAs. However, miR-22-3p showed a conspicuous distribution of miRNA expression.

5 Discussion

5.1 Main finding

In our study cohort, there was no significant difference in ΔΔCq and fold change of miRNA expression between the group with HT and the healthy control group. Although the intended matching of the HT and control groups was not possible due to small sample size, both groups showed no significant differences in age or thyroid function tests and can therefore be regarded as matched.

Based on these sample size limitations the study cannot confirm the data by Martinez-Hernandez and Yamada.

Martinez-Hernandez et al. investigated miRNA expression in thyroid tissue as well as serum. Out of 36 tissue samples (9 HT, 17 GD, 10 controls) 20 were screened using *next generation sequencing* and validated using qRT-PCR. 58 serum samples (14 HT, 22 GD, 22 controls) were validated using qRT-PCR. They found that the circulating levels of 5 miRNAs (miR-21-5p, -96-5p, -142-3p and -301a-3p) were altered in HT. MiR-Let7d, -21-5p, -96-5p, -142-3p and -301-3p expression correlated with thyroid antibody levels.

Yamada et al. investigated miRNA expression in serum in a sample of 64 females (27 HT, 17 GD, 20 controls). The serum samples were first screened for aberrant miRNA expression using microarray technology and validated with qRT-PCR. They found elevated levels of miR-22, -375, 451 in HT and additionally miR-16 in GD.

However, miR-22-3p showed an interesting distribution in the HT group in our study. The ΔΔCq values clustered around +2 and -2, hinting at two distinct subgroups in HT. Upon further analysis, participants with high ΔΔCq values, which represent low miRNA expression, appeared to have higher antibody titers despite the sample sizes being too small to be able to infer further information.

MiR-22-3p is linked to several autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis. It seems to modulate the differentiation and maturation of immune cells, especially dendritic cells and Tregs.(106) In addition to lymphocyte maturation, miR-22, in tandem miR-485-5p, is involved in megakaryopoiesis.(107) Moreover, miR-22 regulates the production of IL-1β and IL-18 by inhibiting the expression of inflammasome proteins.(108)

MiR-22 also inhibits TSH-induced IL-6 receptor upregulation and TSH in turn down-regulates miR-22-3p.(109) Furthermore, T2, the inactive T3 metabolite, has beneficial effects on glucose metabolism ameliorating glucose tolerance and insulin resistance. Some of these effects seem to be mediated through down-regulation of miR-22-3p. Indeed, miR-22-3p is linked to insulin resistance and type 2 diabetes mellitus.(110) Similar to miR-142-5p, it seems to inhibit expression of claudin molecules which play an important role in hiding the antigenic colloid from immune surveillance.(111)
Thus, miR-22-3p can be seen as an important biomarker candidate, also in our study, while expression differences did not reach significant levels.

MiRNAs could help answer the few remaining, but most important, questions in AITD, such as how exactly the risk factors eventually lead to the outbreak of the autoimmune attack. With HT being one of the most archetypal autoimmune disorders it is a very promising candidate. Insights in AITD might change how we see and treat other autoimmune disorders as well. In addition to pathogenetic understanding, miRNAs could be used to differentiate the various subtypes of HT and more importantly guide therapy decisions based on these considerations. In the advent of more modern substitution treatments with liothyronine miRNAs might prove useful for guiding and monitoring therapy modalities, particularly in HT patients with residual symptoms.

MiRNAs provide valuable insights into physiological and pathological processes, but they also have the potential to serve as modern biomarkers. Nevertheless, there are still open questions about the fundamentals of this group of molecules:
MiRNAs are present in very low concentrations, especially considering the overwhelming abundance of possible target mRNAs. Furthermore, translational repression reduces translation about 20% in most cases. Considering these factors, translational repression by miRNAs can be considered to have little impact. However, knock-out of Dicer or Drosha in mice leads to non-viable embryos illustrating the necessity of miRNA dependent modulation of translation. Knock-out of single miRNAs can render embryos non-viable or with severe disorders. In addition to miRNAs constituting about 3% of the human genome, the degree of conservation of these sequences implies a much more profound role that can be explained by concentrations alone.(86, 92)

Regarding their role as potential biomarkers, there are four qualities a good biomarker should provide to warrant clinical usage: specificity, sensitivity, stability and a non-invasive way to obtain the sample.(92) While circulating miRNAs fulfill the latter qualities, they still show deficits in diagnostic accuracy as of yet.

Strengths of the study

Blood sampling was done in a standardized manner (in the morning after an overnight fast) to account for potential diurnal variations. Furthermore, analysis of the samples was done immediately with reducing potential delays for the prevention of possible sample degradation due to freezing and thawing cycles. Another strong point was the prospective recruitment process, which allowed for more representative sampling of the participants. Lastly, the recent publication about miRNAs in thyroid diseases by Trummer et al. was aided by preliminary work done for this diploma thesis.

Limitations of the study

A certain selection bias in the current cohort might not be negligible due to the recruitment in an outpatient clinic. Patient recruitment also stopped prior to the expected sample size because of the COVID pandemic.
Originally, two patient visits were planned to investigate changes in several clinical and biochemical parameters. Due to restrictions caused by the outbreak of the Covid-19 pandemic, the second patient visits were not feasible. Therefore, no prognostic data on clinical development, which was assessed with the questionnaires, sonograms, and laboratory analyses, were available.
Finally, no reference genes for miRNA normalization were available. So, potential differences may have been missed. In addition to these reference genes, miR-Let7d was not available because of supply difficulties, even though this miRNA had the most significant alteration in expression in Martinez-Hernandez et al.

Overall, studies on miRNA are methodically rather heterogenous in regards to sample tissues (plasma, serum, PBMC, thyroid tissue), miRNA carriers (PBMC, exosomes, Ago), preservation methods (frozen, paraffin embedded) or methods (NGS, microarray, RT-

qPCR).(92, 100) Comparisons of even the same miRNA are therefore difficult to interpret between two studies.

5.2 Conclusion

In summary, our study could not confirm the differential expression presented by Martinez-Hernandez and Yamada, although its power was limited due to a small sample size with yet an interesting miRNA candidate. As prospective miRNA studies in general have rather few participants, this is a challenge that should be tackled in future research. However, the standardization of miRNA assessment is probably biggest challenge in miRNA studies. To achieve a meaningful degree of comparability between studies, a more standardized approach has to be designed and broadly applied.

A deeper understanding of HT might be useful to gain insights into the pathogenesis for AITD and autoimmune disorders in general, one of the greatest challenges in modern medicine. However, further high-quality research is needed to facilitate broader use of miRNAs in clinical practice.

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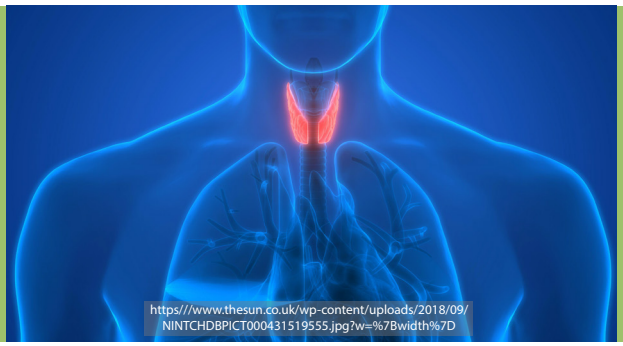
Appendix

HashmiR - Studie
Schilddrüsen-MicroRNAs

Medizinische Universität Graz

Was macht die Schilddrüse?

Sie produziert die Schilddrüsenhormone **T3 & T4**. Diese regen den Stoffwechsel an und spielen auch bei der **geistigen und körperlichen Reifung** eine wichtige Rolle.
Sie regulieren die **Herzfunktion, Körpertemperatur, Energieumsatz, Blutzucker, Blutfette**.

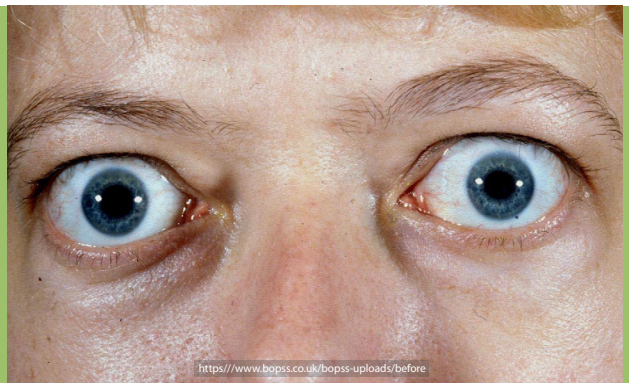


Was sind Autoimmunthyreopathien?

Das sind Erkrankungen der Schilddrüse, die auf autoimmunen Prozessen basieren. Dabei greift das Immunsystem körpereigenes Gewebe an und führt über eine Entzündung zu einer Über- oder Unterfunktion der Schilddrüse.

Was machen AITDs?

Sie führen über eine gehemmte Produktion von Schilddrüsenhormonen (Hypothyreose) zu Symptomen wie **Depressivität, Gewichtszunahme, Antriebslosigkeit oder Zyklusstörungen.**
Es kann allerdings auch zu einer gesteigerten Produktion (Hyperthyreose) kommen, diese führt zu **Herzrasen, Gewichtsabnahme, Reizbarkeit** oder **hervortretenden Augen.**



Was sind Micro-RNAs?

Micro-RNAs oder kurz miRNAs sind kurze RNA-Abschnitte, also Moleküle, die unserer DNA ähneln. miRNAs finden sich in Zellen oder im Blutkreislauf und regulieren verschiedenste zelluläre Abläufe indem sie meist hemmend ein diese eingreifen.

Ziel der Studie:

Neue Studien haben gezeigt, dass ein Zusammenhang zwischen bestimmten miRNAs und AITDs herrscht.
Unser Ziel ist es diesen für diagnostische Zwecke zu nutzen, um dadurch zuverlässigere Diagnosen bei Schilddrüsenerkrankungen stellen zu können.

Auskunft:
Herr Lukas Fank, Endokrinologie-Laborplattform
0316-385-12517 / LukasOtmarGerhard.Fank@klinikum-graz.at

Anmeldung:
Endokrinologie-Ambulanz
0316-385-12303 (täglich von 10:00-12:00)

v1.0 vom 15.11.2018

Medizinische Universität Graz

SDF

HashmiR

Von Klinikpersonal auszufüllen

Subject-ID	___ ___ ___ ___ ___ ___
Initialen des Subjects	

Richtlinien zum Ausfüllen des Source Data Forms

- ➔ Bitte alle Informationen leserlich mit Kugelschreiber ausfüllen.
- ➔ Text sollte auch für Nichtmediziner/innen verständlich sein.
- ➔ Keine Abkürzungen oder Symbole benutzen. Wenn Abkürzungen verwendet werden müssen, müssen diese erklärt werden.
- ➔ Keine Felder oder Fragen unbeantwortet lassen.
- ➔ □ Boxen zum ankreuzen bitte folgendermaßen ausfüllen ☒

- ➔ 1 2 3 Numerische Felder müssen mit Zahlen ausgefüllt werden. Keine Felder freilassen oder ausstreichen --- oder /

- ➔ A B C Character fields müssen Buchstaben enthalten. Felder nicht freilassen..

- ➔ Text Textfelder müssen mit Text gefüllt werden.

- ➔ Wenn Datenfelder nicht ausgefüllt werden können bitte „ND“ für “Not Done” einfügen. Wenn nötig, bitte eine Erklärung abgeben warum das Feld nicht ausgefüllt werden konnte.

- ➔ Wen ein exaktes Datum nicht bekannt ist, soweit wie möglich ausfüllen.
  zB N A/ 1 1/ 1 1

- ➔ Korrekturen bitte folgendermaßen durchführen. Durch die falschen Daten eine geraden Strich durchziehen, richtige Daten danebenschreiben. Jede Korrektur mit Datum und Initialen des/der Korrigierenden versehen. Wenn nötig, eine Erklärung anfügen. Beispiel:

  02 SK02-Feb-2011

  ~~0 3~~/ 1 1/ 1 1

Visitenplan

Visite 1

Blutproben
Urinprobe
SDF
Sonographie
DXA
HRpqCT bei Risikopatienten

Visite 2 (nach 1 Jahr)

Blutproben
Urinprobe
SDF
Sonographie
DXA
HRpqCT bei Risikopatienten

Visite 1 Datum der Visite _ _ /_ _/_ _ _ _

VON ARZT/ÄRZTIN AUSZUFÜLLEN

EINVERSTÄNDNISERKLÄRUNGEN (INFORMED CONSENTS)		
Patienten IC vor Setzen von studienrelevanten Massnahmen unterschrieben?	ja ☐	nein ☐
Endoregister IC vor Setzen von studienrelevanten Massnahmen unterschrieben?	ja ☐	nein☐
Initialen des Arztes		
Datum des Erhaltes der Informed consents	___ ___ /___ ___ /___ ___ ___ ___ (tt/mm/jjjj)	

EINSCHLUSSKRITERIEN	ja	nein	Datenquelle
Alter 18 bis 70 Jahre (beides inklusive)	☐	☐	
Autoimmunthyreopathie	☐	☐	

AB: Arztbrief, ME: Medocs, PA: Patientenakte

AUSSCHLUSSKRITERIUM (muss mit "nein" beantwortet werden)	ja	nein
Schwangerschaft	☐	☐

CLINICAL ACTIVITY SCORE (CAS) TEIL 1		
Endokrine Orbitopathie ("Mb. Basedow"="Graves' disease")	ja ☐	nein ☐
aktiv ja/nein	ja ☐	nein ☐
Schweregrad (NOSPECS-Klassifikation)	☐ 0 (keine Zeichen oder Symptome)	
	☐ 1 (nur Zeichen, keine Symptome)	
	☐ 2 (Weichteilbeteiligung)	
	☐ 3 (Exophthalmus)	
	☐ 4 (Muskelveränderung)	
	☐ 5 (Hornhautkomplikation	

VON PFLEGE im Dekurs AUSZUFÜLLEN

ANTHROPOMETRISCHE DATEN	
Körpergewicht (ohne Schuhe und Jacke)	__ __ __,__ kg
Größe (ohne Schuhe)	__ __ __,__ cm
BMI	__ __,__ kg/m^2
Hüftumfang (in stehender Position über dem großen Trochanter)	__ __,__ cm
Taillenumfang (in stehender Position nach leichtem Ausatmen)	__ __,__ cm

VON DIPLOMAND AUSZUFÜLLEN

DEMOGRAPHISCHE DATEN			
Geschlecht	männlich ☐	weiblich ☐	
Geburtsdatum (tt/mm/jjjj)	_ _/_ _/_ _ _ _	Alter	_____ Jahre

SUBGRUPPEN (Tel 83386)	ja	nein
Osteoporose-Risiko erhöht	☐	☐
ausgeprägte klinische Schilddrüsensymptomatik	☐	☐
DXA T-Wert < -1	☐	☐
Hyperthyreose	☐	☐

MEDIZINISCHE ANAMNESE/BEGLEITERKRANKUNGEN (Autoimmunthyreopathie inklusive)

Wenn d. Patient/in relevante Erkrankungen in der Vergangenheit oder jetzt aufweist, bitte unten eintragen

Erkrankung	Beginn tt/mm/jj	Ende tt/mm/jj	fortlaufend
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐

BEGLEITMEDIKATION

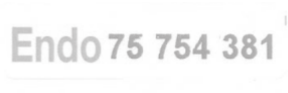
Jede Behandlung innerhalb der letzten 6 Monate vor der Visite

Handelsname	Indikation	Dosierung	Frequenz	Beginn tt/mm/jj	Ende tt/mm/jj	fortlaufend
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐

PROBEN			
Serum mit Gel 8 ml	2 x	ja ☐	nein ☐
Lithium Heparin 8 ml	1 x	ja ☐	nein ☐
EDTA 3 ml* (Eisbad)	1 x	ja ☐	nein ☐
EDTA 3 ml	2 x	ja ☐	nein ☐
Urin 8 ml	1 x	ja ☐	nein ☐
Wenn nein, welche Probe wurde nicht entnommen und warum: […]			

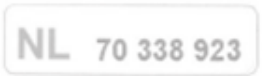
Laborbelegsnummerpickerl V1 LB2

Laborbelegsnummerpickerl V1 Endo



Laborbelegsnummerpickerl V1 Rheuma/Immuno

Laborbelegsnummerpickerl V1 Nuklearmedizin



BILDGEBUNG

DXA Messung	ja ☐	nein ☐
HR-pQCT Messung	ja ☐	nein ☐
Schilddrüsensonographie, -elastographie	ja ☐	nein ☐
Wenn nein, warum nicht: […]		

Visite 2 Datum der Visite _ _ /_ _/_ _ _ _

VON ARZT/ÄRZTIN AUSZUFÜLLEN

EINSCHLUSSKRITERIEN	ja	nein	Datenquelle
Alter 18 bis 70 Jahre (beides inklusive)	☐	☐	
Autoimmunthyreopathie	☐	☐	

AB: Arztbrief, ME: Medocs, PA: Patientenakte

AUSSCHLUSSKRITERIUM (muss mit "nein" beantwortet werden)	ja	nein
Schwangerschaft	☐	☐

CLINICAL ACTIVITY SCORE (CAS)		
Endokrine Orbitopathie ("Mb. Basedow"="Graves' disease")	ja ☐	nein ☐
aktiv ja/nein	ja ☐	nein ☐
Schweregrad (NOSPECS-Klassifikation)	☐ 0 (keine Zeichen oder Symptome)	
	☐ 1 (nur Zeichen, keine Symptome)	
	☐ 2 (Weichteilbeteiligung)	
	☐ 3 (Exophthalmus)	
	☐ 4 (Museklveränderung)	
	☐ 5 (Hornhautkomplikation	

VON PFLEGE am Laufzettel AUSZUFÜLLEN

ANTHROPOMETRISCHE DATEN	
Körpergewicht (ohne Schuhe und Jacke)	__ __ __,__ kg
Größe (ohne Schuhe)	__ __ __,__ cm
BMI	__ __,__ kg/m^2
Hüftumfang (in stehender Position über dem großen Trochanter)	__ __,__ cm
Taillenumfang (in stehender Position nach leichtem Ausatmen)	__ __,__ cm

VON DIPLOMAND AUSZUFÜLLEN

DEMOGRAPHISCHE DATEN			
Geschlecht	männlich ☐	weiblich ☐	
Geburtsdatum (tt/mm/jjjj)	_ _/_ _/_ _ _ _	Alter	_____ Jahre

SUBGRUPPEN (Tel 83386)	ja	nein
Osteoporose-Risiko erhöht	☐	☐
ausgeprägte klinische Schilddrüsensymptomatik	☐	☐
DXA T-Wert < -1	☐	☐
Hyperthyreose	☐	☐

MEDIZINISCHE ANAMNESE/BEGLEITERKRANKUNGEN (zusätzlich zu denen aus Visite 1)

Wenn d. Patient/in relevante Erkrankungen in der Vergangenheit oder jetzt aufweist, bitte unten eintragen

Erkrankung	Beginn tt/mm/jj	Ende tt/mm/jj	fortlaufend
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐
	__ __/__ __/__ __	__ __/__ __/__ __	☐

BEGLEITMEDIKATION (zusätzlich zu Visite 1)

Jede Behandlung innerhalb der letzten 6 Monate vor der Visite

Handelsname	Indikation	Dosierung	Frequenz	Beginn tt/mm/jj	Ende tt/mm/jj	fortlaufend
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐
				_ _/_ _/_ _	_ _/_ _/_ _	☐

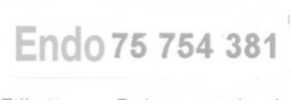
Änderungen zu Visite 1

PROBEN			
Serum mit Gel 8 ml	2 x	ja ☐	nein ☐
Lithium Heparin 8 ml	1 x	ja ☐	nein ☐
EDTA 3 ml* (Eisbad)	1 x	ja ☐	nein ☐
EDTA 3 ml	2 x	ja ☐	nein ☐
Urin 8 ml	1 x	ja ☐	nein ☐
Wenn nein, welche Probe wurde nicht entnommen und warum: […]			

*1 Röhrchen kühlen

Laborbelegsnummerpickerl V2 LB2

Laborbelegsnummerpickerl V2 Endo



Laborbelegsnummerpickerl V2 Rheuma/Immuno

Laborbelegsnummerpickerl V2 Nuklearmedizin

BILDGEBUNG		
DXA Messung	ja ☐	nein ☐
HR-pQCT Messung	ja ☐	nein ☐
Schilddrüsensonographie, -elastographie	ja ☐	nein ☐
Wenn nein, warum nicht: […]		

☐ Kontrolle auf Vollständigkeit des Datenblattes

Datum: (tt/mm/jjjj)	_ _/_ _/_ _ _ _
Unterschrift Prüfarzt/Prüfärztin:	

Drop Out/ EXCL

Datum Drop Outs/ EXCL	_ _ /_ _ / _ _ _ _		
Gründe für Drop Outs / EXCL			
Unterschrift Mitarbeiter/in + Datum:			

Kommentare – UP-DATES – ADVERSE EVENTS

Datum	**Zeit** (hh:mm)	
_ _/_ _/_ _ _ _	_ _ :_ _	
_ _/_ _/_ _ _ _	_ _ :_ _	
_ _/_ _/_ _ _ _	_ _ :_ _	
_ _/_ _/_ _ _ _	_ _ :_ _	

HashmiR-Studie	**Fragebogen**	
PatientInnenID: _____	**Datum (tt/mm/jjjj):** __ __/__ __/__ __ __ __	**Geburtsdatum (tt/mm/jjjj):** __ __/__ __/__ __ __ __

Vergrößerung der Schilddrüse (Struma)	ja ☐	nein ☐
Bestand schon eine Schilddrüsenerkrankung? Seit wann (Jahr)?	ja ☐	nein ☐
Gewichtsveränderung? .....kg seit wann.....	ja ☐	nein ☐
Kürzliche Knochenbrüche im Rahmen von Osteoporose	ja ☐	nein ☐
Zyklusstörungen	ja ☐	nein ☐
Unerfüllter Kinderwunsch	ja ☐	nein ☐
Andere Symptome? Welche.....	ja ☐	nein ☐
Schilddrüsenunterfunktion in der Familie	ja ☐	nein ☐
Schilddrüsenüberfunktion in der Familie	ja ☐	nein ☐
Verwandtschaftsgrad? Wenn ja, welche Erkrankung:		

Merkmale	**Trifft nicht zu** **Trifft ganz zu**
Gesteigerter Appetit und/oder Heißhunger	0 |.....|.....|.....|.....|.....|5
Obstipationen/Verstopfung	0 |.....|.....|.....|.....|.....| 5
Häufiger Stuhlgang oder Durchfälle?	0 |.....|.....|.....|.....|.....|5
Verstärktes Kältegefühl	0 |.....|.....|.....|.....|.....| 5
Hitzeintoleranz oder häufiges Schwitzen	0 |.....|.....|.....|.....|.....|5
Zittern	0 |.....|.....|.....|.....|.....| 5
Schlaflosigkeit	0 |.....|.....|.....|.....|.....|5
Müdigkeit	0 |.....|.....|.....|.....|.....| 5
Muskelschwäche	0 |.....|.....|.....|.....|.....|5
Langsamer Herzschlag (Bradykardie)	0 |.....|.....|.....|.....|.....| 5
Herzrasen (Tachykardie)	0 |.....|.....|.....|.....|.....|5
geschwollene Finger oder sonstige Ödeme	0 |.....|.....|.....|.....|.....| 5
Trockene und/oder schuppige Haut	0 |.....|.....|.....|.....|.....|5
Raue, brüchige Stimme	0 |.....|.....|.....|.....|.....| 5
Haarausfall	0 |.....|.....|.....|.....|.....|5

Vielen Dank für Ihre Mitarbeit!

Laufzettel HashmiR-Studie Patientennummer HA __ __ __ __

„HashmiR-Studie“: Ablauf

Vielen Dank für Ihre Teilnahme an der HashmiR Studie!

Im Rahmen der HashmiR-Studie bitte Folgendes durchführen:

Wartebereich vor Ambulanzschalter	Nach der Anmeldung bitte im Wartebereich Platz nehmen. Sie werden in U2 aufgerufen
U2	• Einverständniserklärung lesen und unterzeichnen • Fragebogen ausfüllen (evtl. schon im Warteraum) • Schilddrüsenuntersuchung
U1	• Größe, Gewicht, Hüft- und Taillenumfang werden gemessen • Sie erhalten einen Harnbecher. Bitte eine Harnprobe abgeben und den Becher auf U1 zurückbringen.
(Wartebereich vor U5)	Falls Sei einen zeitnahen Termin zur Knochendichtemessung (NUR wenn von Arzt/Ärztin angeordnet) haben nehmen Sie bitte im Wartebereich vor U5 Platz, Sie werden aufgerufen.
(U5)	Knochendichtemessung DXA evtl. HRpQCT Termin (Spezialmessung, NUR wenn von Arzt/Ärztin angeordnet) geben lassen
Blutabnahme 1. Stock	Falls Sie keinen Knochendichtemessungstermin haben, bitte weiter zur Blutabnahme Diese ist bis 13:00 Uhr möglich  Sie nehmen den mittleren Lift (dunkelgrauer Punkt in der Abbildung) und fahren in den 1. Stock. Dann gehen Sie nach links, bis Sie auf der linken Seite den Wartebereich für die Blutabnahme sehen. Bitte melden Sie sich am Schalter an und geben Sie die 3 Laborzuweisungsbelege, die Einverständniserklärung für genetische Untersuchungen und die gelben Patientenetiketten ab.
Sie erhalten einen Arztbrief mit allen Befunden per Post bzw. Ihr/e Hausarzt/ärztin elektronisch zugeschickt	

Vielen Dank! Sie unterstützen damit die Forschung an der Medizinischen Universität Graz.

Version 1.1 vom 04.06.2019

LKH – Universitätsklinikum Graz
Universitätsklinik für Innere Medizin
Klinische Abteilung für Endokrinologie und Diabetologie
Leiter: Univ.Prof.Dr. Thomas Pieber

KAGes

Zuweisung für

KNOCHENDICHTEMESSUNG

Anmeldung unter

Tel.: 0316/385/13386 **Fax: 0316/14845**

PATIENTIN:

KONTAKTPERSON/ TEL.:

TERMIN für HRpQCT (wird nach Dexa vereinbart):

Datum:_____

Zeit:_____

- ○ **HRpQCT**
- ○ **DXA**
  - o Schenkelhals
  - o LWS
  - o TBS

- ○ **STUDIE**
  - o Name der Studie: HASHMIR
  - o EK Nr: 31-118 ex 18/19
  - o Verrechnung als x Studie o ambulanter Besuch
  - o StudienleiterIn Univ.Prof.in.Dr.in Barbara Obermayer-Pietsch

ProbandInneninformation und Einwilligungserklärung zur Teilnahme an der Beobachtungsstudie

[1]miRNA-Signaturen bei [2]Immunthyreopathien – die "HashmiR" – Studie

Sehr geehrte Frau/ sehr geehrter Herr

Name ……

Geburtsdatum ……..

Wir laden Sie ein, an der oben genannten Studie teilzunehmen. Die Aufklärung darüber erfolgt in einem ausführlichen ärztlichen Gespräch.

Ihre Teilnahme an dieser Beobachtungsstudie erfolgt freiwillig. Sie können jederzeit ohne Angabe von Gründen aus der Studie ausscheiden. Die Ablehnung der Teilnahme oder ein vorzeitiges Ausscheiden aus dieser Studie hat keine nachteiligen Folgen für Ihre medizinische Betreuung.

Klinische Studien sind notwendig, um verlässliche neue medizinische Forschungsergebnisse zu gewinnen. Unverzichtbare Voraussetzung für die Durchführung einer klinischen Studie ist jedoch, dass Sie Ihr Einverständnis zur Teilnahme an dieser klinischen Studie schriftlich erklären.

Bitte lesen Sie den folgenden Text als Ergänzung zum Informationsgespräch mit Ihrem Arzt/Ärztin sorgfältig durch und zögern Sie nicht Fragen zu stellen.

Bitte unterschreiben Sie die Einwilligungserklärung nur

- wenn Sie Art und Ablauf der klinischen Studie vollständig verstanden haben,

- wenn Sie bereit sind, der Teilnahme zuzustimmen und

- wenn Sie sich über Ihre Rechte als TeilnehmerIn an dieser klinischen Studie im Klaren sind.

Zu dieser Studie, sowie zur ProbandInneninformation und Einwilligungserklärung wurde von der zuständigen Ethikkommission eine befürwortende Stellungnahme abgegeben.

Ziele dieser Studie sind, miRNAs als neue [3]Biomarker für die Diagnose und Vorhersage von Schilddrüsenerkrankungen (sogenannten Autoimmunthyreopathien) zu

[1]miRNA-Signatur: Kombination verschiedener microRNAs (der DNA ähnelnde, kurze Moleküle, die die Herstellung von Genprodukten beeinflussen), [2]Immunthyreopathie/Autoimmunthyreopathie: durch das (eigene) Immunsystem hervorgerufene Schilddrüsenerkrankung; [3]Biomarker: Messwert der Auskunft über einen bestimmten Zustand des Körpers gibt; [4]DNA: Träger der Erbinformation, [5]Basedow: eine Autoimmune Schilddrüsenerkrankung mit Schilddrüsenüberfunktion, [6]HRpQCT: hochauflösende CT-Untersuchung, die die Mikrostruktur des Knochens zeigt; [7]Genregulation: Veränderung der Menge an gebildeten Proteinen (=Eiweiße) [8]Zirkulierende miRNA: im Blutstrom befindliche miRNA

untersuchen und mit deren Hilfe in Zukunft zuverlässigere diagnostische Methoden zu entwickeln und den Einfluss von bestimmten Veränderungen in der [4]DNA auf das Risiko eines neuerlichen Auftretens der Autoimmunthyreopathie [5]Basedow zu untersuchen.

Im Rahmen dieser Studie sollen miRNA-Spiegel im Blut analysiert werden. Des Weiteren ist die Untersuchung von relevanten Genen, die bei diesen Schilddrüsenerkrankungen eine Rolle spielen, und bei einigen PatientInnen die Messung der Knochenfeinstruktur mittels Knochendichtemessung und [6]HRpQCT-Untersuchung vorgesehen.

Wir erwarten im Zuge der Studie Informationen darüber, ob im Blut zirkulierenden miRNAs mit bestimmten Schilddrüsenerkrankungen im Zusammenhang stehen und ob es einen Zusammenhang mit deren Schweregrad gibt.

Ablauf dieser Studie: Diese Studie wird am Universitätsklinikum Graz durchgeführt, es werden insgesamt 540 Personen daran teilnehmen. Ihre Teilnahme an dieser Studie wird im Rahmen von zwei ambulanten Untersuchungen im Abstand von 1 Jahr stattfinden.

<u>Folgende Maßnahmen werden routinemäßig durchgeführt, und die Ergebnisse für die Studie verwendet:</u>

a) Bestimmung von Blutparametern aus insgesamt 30 ml Blut, das entspricht 3 Esslöffeln Blut.
    a. Blutbild (3 ml Blut) bei jeder Visite
    b. Schilddrüsenparameter, Vitamin D und Knochenstoffwechsel (1 x 8 ml Serum, 1x 3 ml EDTA, gesamt 11 ml Blut) (bei jeder Visite)
    c. Elektrolyte, Leber- und Nierenparameter (8 ml Blut) (bei jeder Visite)
    d. Entzündungsparameter (8 ml Blut)
b) Bestimmung von Jod im Harn (bei jeder Visite): (8 ml Harn)
c) Ultraschalluntersuchung der Schilddrüse
d) Bei klinischem Osteoporoserisiko Knochendichtemessung (DXA und HRpQCT)

<u>Folgende Maßnahmen werden ausschließlich aus Studiengründen durchgeführt:</u>

Es werden bei jeder Ihrer ambulanten Untersuchungen zu Forschungszwecken zusätzlich zur Routine-Blutabnahme 19 ml Blut abgenommen, das entspricht 2 Esslöffeln Blut.

a) Genetische Untersuchung für Schilddrüsenerkrankungen: Aus der EDTA-Probe werden Blutzellen isoliert, daraus Erbmaterial (DNA) gewonnen und verschiedene Varianten in Genen des Immunsystems untersucht. Zu diesem Zweck wird ein EDTA-Röhrchen (3 ml Blut) abgenommen.

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b) Aus der Serumprobe wird weiteres Erbmaterial isoliert (RNA) und [7]genregulatorische Veränderungen (z.B. [8]zirkulierenden miRNAs) untersucht. Zu diesem Zweck wird bei jeder ambulanten Untersuchung ein Serumröhrchen (8 ml Blut) abgenommen.
c) Aus einer Blutprobe (8 ml Lithium-Heparin) werden die Anzahl und Art von Immunzellen bei jeder Visite bestimmt.
d) Ein Fragebogen zur Bestimmung des klinischen Schweregrades der Erkrankung wird vorgelegt.

Diese Untersuchungen werden zu wissenschaftlichen Zwecken durchgeführt. Eine Übermittlung der Gesamtergebnisse der Studie ist nach deren Abschluss gerne möglich, individuelle Ergebnisse können nur in Ausnahmefällen mitgeteilt werden.

Alle Analysen werden an der Medizinischen Universität Graz, Univ. Klinik für Innere Medizin, Klinische Abteilung für Endokrinologie und Diabetologie, Endokrinologie-Laborplattform (Leiterin: Univ. Prof.in Dr.in Barbara Obermayer-Pietsch) durchgeführt.

Die Proben werden laut Vereinbarung der klinischen Abteilung für Endokrinologie und Diabetologie aufbewahrt. Die Biobank-Einverständniserklärung wird separat vorgelegt.

Die Proben werden laut Biobankvereinbarung zwischen Biobank und Klinischer Abteilung für Endokrinologie und Diabetologie unter der Verantwortlichkeit der Laborleiterin Univ.-Prof.in Dr.in Barbara Obermayer-Pietsch aufbewahrt. Sollten Sie mit der Aufbewahrung des Materials nicht einverstanden sein, wird das Material nach Durchführung der in diesem Projekt geplanten Untersuchungen unter Kontrolle der Projektleiterin vernichtet. Der Schutz vor dem Zugriff Unbefugter ist nach dem österr. Gentechnikgesetz sichergestellt. Es erfolgt eine Anonymisierung und Kodierung der Proben mit Verschlüsselung der personenbezogenen Daten. Der Schlüsselcode liegt bei der Studienleiterin für die kommenden 20 Jahre auf.

Die wissenschaftlichen Untersuchungen und Experimente werden alle unter Einhaltung des geltenden Gentechnikgesetzes durchgeführt. Alle Arbeiten während der Studie unterliegen den Bestimmungen der guten klinischen Praxis (GCP) sowie den ethischen Prinzipien, beschlossen in der Deklaration von Helsinki.

Nutzen einer Teilnahme an dieser Studie: Es ist möglich, dass Sie durch Ihre Teilnahme an dieser Studie keinen direkten Nutzen für Ihre Gesundheit haben. Jedoch können durch die Analyse der zirkulierenden miRNAs Erkenntnisse zu Autoimmunthyreopathien gewonnen werden. Diese können in Zukunft als neue Biomarker zur Diagnose eingesetzt werden.

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Darüber hinaus ist es das Ziel dieser Studie, neue wissenschaftliche Erkenntnisse zu gewinnen, die in Zukunft sowohl anderen PatientInnen als auch gesunden Personen von Nutzen sein können.

Mögliche Risiken, Beschwerden und Begleiterscheinungen: Da die Studie im Verlauf einer bereits bestehenden Erkrankung und Behandlung ohne neue Medikamente stattfindet, sind keine Beschwerden und Risiken die vor, während oder nach der Untersuchung entstehen können, möglich. Über mögliche grundsätzliche mit Krankheiten oder der Behandlung in Zusammenhang stehende Beschwerden werden sie von Ihrem/Ihrer behandelnden Arzt/Ärztin informiert.

Die Untersuchungen, die ausschließlich im Rahmen der Studie durchgeführt werden, können theoretisch zu geringen zusätzlichen Beschwerden führen. So kann es zu leichten Schmerzen oder zu Blutergüssen bei der Blutabnahme kommen.

Zusätzliche Einnahme von Arzneimitteln: Für die Auswertung der Ergebnisse dieser Studie ist es sehr wichtig, dass die Forschungspersonen über alle von Ihnen eingenommenen Medikamente informiert werden. Wir bitten Sie daher, die Einnahme aller Arzneimittel sowie aller Vitamin- und Mineralstoffpräparate (Präparat Einnahme, seit wann und in welcher Dosis) Ihrem/er Studienarzt/ärztin mitzuteilen, da diese für die Ergebnisse bedeutend sein können. Eine Veränderung ihrer Behandlung ist jedoch nicht erforderlich.

Auswirkungen der Teilnahme an der klinischen Studie auf die Lebensführung und sich daraus ergebende Verpflichtungen: Da die für die Studie notwendigen Maßnahmen während Routinekontrollen durchgeführt werden ist kein zusätzlicher Aufwand für die Studie erforderlich.

Auftreten von Symptomen, Begleiterscheinungen und/oder Verletzungen: Die vorliegende Studie bedingt keine Symptome, Begleiterscheinungen und/oder Verletzungen. Sollten trotzdem im Verlauf der klinischen Studie irgendwelche Symptome, Begleiterscheinungen oder Verletzungen auftreten, ersuchen wir Sie, diese Ihrem/er Arzt/Ärztin mitzuteilen.

Vorzeitige Beendigung der klinischen Studie: Sie können jederzeit auch ohne Angabe von Gründen, Ihre Teilnahmebereitschaft widerrufen und aus der klinischen Studie ausscheiden, ohne dass Ihnen dadurch irgendwelche Nachteile für Ihre weitere medizinische Betreuung entstehen.

Ihr/e Studienarzt/ärztin wird Sie über alle neuen Erkenntnisse, die im Rahmen dieser klinischen Studie bekannt werden und für Sie wesentlich werden könnten, umgehend informieren. Auf dieser Basis können Sie dann Ihre Entscheidung zur **weiteren** Teilnahme an dieser klinischen Studie neu überdenken.

Es ist aber auch möglich, dass Ihr/e Studienarzt/ärztin entscheidet, Ihre Teilnahme

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an der klinischen Studie vorzeitig zu beenden, ohne vorher Ihr Einverständnis einzuholen.

Verwendung der im Rahmen dieser klinischen Studie gesammelten Daten: Bei den Daten, die über Sie im Rahmen dieser klinischen Studie erhoben werden, ist grundsätzlich zu unterscheiden zwischen

1) jenen personenbezogenen Daten, anhand derer Sie direkt identifizierbar sind (z.B. Name, Geburtsdatum, Adresse...) und
2) pseudonymisierten (verschlüsselten) Daten, bei denen alle Informationen, die direkte Rückschlüsse auf Ihre Identität zulassen, durch einen Code (z. B. eine Zahl) ersetzt werden.

Der Code wird von den verschlüsselten Datensätzen streng getrennt und nur an Ihrem Prüfzentrum aufbewahrt. Zugang zu Ihren nicht verschlüsselten Daten haben der/die Studienarzt/ärztin und andere MitarbeiterInnen des Prüfzentrums, die an der klinischen Studie oder Ihrer medizinischen Versorgung mitwirken. Die Daten sind gegen unbefugten Zugriff geschützt. Zusätzlich können autorisierte und zur Verschwiegenheit verpflichtete Beauftragte der Medizinischen Universität Graz, sowie Beauftragte von In- und/oder ausländischen Gesundheitsbehörden und jeweils zuständige Ethikkommissionen in die nicht verschlüsselten Daten Einsicht nehmen, soweit dies für die Überprüfung der ordnungsgemäßen Durchführung der klinischen Studie notwendig bzw. vorgeschrieben ist. Diese Personen unterliegen einer strengen Geheimhaltungspflicht.

Eine Weitergabe der Daten erfolgt nur in verschlüsselter Form. Auch für etwaige Publikationen werden nur die verschlüsselten Daten verwendet.

Sie können Ihre Einwilligung zur Erhebung Ihrer Daten jederzeit widerrufen. Nach Ihrem Widerruf werden keine weiteren Daten mehr über Sie erhoben. Die bis zum Widerruf erhobenen Daten können allerdings weiter im Rahmen dieser klinischen Studie verwendet werden.

Aufgrund der gesetzlichen Vorgaben haben Sie außerdem, sofern dies nicht die Durchführung der klinischen Studie beeinträchtigt, das Recht auf Einsicht in die von Ihnen erhobenen Daten und die Möglichkeit der Berichtigung, falls Sie Fehler feststellen.

Sie haben auch das Recht, bei der österreichischen Datenschutzbehörde eine Beschwerde über den Umgang mit Ihren Daten einzubringen (www.dsb.gv.at).

[1]miRNA-Signatur: Kombination verschiedener microRNAs (der DNA ähnelnde, kurze Moleküle, die die Herstellung von Genprodukten beeinflussen), [2]Immunthyreopathie/Autoimmunthyreopathie: durch das (eigene) Immunsystem hervorgerufene Schilddrüsenerkrankung; [3]Biomarker: Messwert der Auskunft über einen bestimmten Zustand des Körpers gibt; [4]DNA: Träger der Erbinformation, [5]Basedow: eine Autoimmune Schilddrüsenerkrankung mit Schilddrüsenüberfunktion, [6]HRpQCT: hochauflösende CT-Untersuchung, die die Mikrostruktur des Knochens zeigt; [7]Genregulation: Veränderung der Menge an gebildeten Proteinen (=Eiweiße) [8]Zirkulierende miRNA: im Blutstrom befindliche miRNA

Sämtliche Personen, die Zugang zu Ihren verschlüsselten und nicht verschlüsselten Daten erhalten, unterliegen im Umgang mit den Daten dem österreichischen Datenschutzgesetz in seiner gültigen Fassung sowie der Datenschutz-Grundverordnung (DSGVO).

Auch die Dauer der Speicherung Ihrer Daten ist durch Rechtsvorschriften geregelt.

Falls Sie Fragen zum Umgang mit Ihren Daten in dieser klinischen Studie haben, wenden Sie sich zunächst an Ihren Studienarzt/ärztin. Dieser kann Ihr Anliegen ggf. an die Personen, die am Prüfzentrum für den Datenschutz verantwortlich sind, weiterleiten.

Datenschutzbeauftragte/r der Medizinischen Universität Graz:

Koordinationsstelle Datenschutz

email: datenschutz@medunigraz.at

Datenschutzbeauftragte/r der KAGES:

Email: datenschutz@kages.at

Entstehende Kosten/Vergütung: Durch Ihre Teilnahme an dieser klinischen Studie entstehen für Sie keine zusätzlichen Kosten und es erfolgt keine finanzielle Vergütung.

Kontaktperson für Fragen oder Widerrufe: Für weitere Fragen im Zusammenhang mit dieser klinischen Studie stehen Ihnen Ihr/e Studienarzt/ärztin und seinen/ihre MitarbeiterInnen gern zur Verfügung. Auch Fragen, die Ihre Rechte als PatientIn und TeilnehmerIn an dieser klinischen Studie betreffen, werden Ihnen gerne beantwortet. Bitte wenden Sie sich auch an folgende Personen falls Sie Ihre Teilnahme widerrufen wollen, mit der Probenaufbewahrung nicht mehr einverstanden sind oder die Studie vorzeitig beenden möchten.

Name der Kontaktperson: Univ.-Prof.^{in} Dr.^{in} med. univ. Barbara Obermayer-Pietsch
Ständig erreichbar unter: 0316-385-12383

Name der Kontaktperson: Mag^{a}. Dr^{in}. Natascha Schweighofer
Ständig erreichbar unter: 0316-385-12383

[1]miRNA-Signatur: Kombination verschiedener microRNAs (der DNA ähnelnde, kurze Moleküle, die die Herstellung von Genprodukten beeinflussen), [2]Immunthyreopathie/Autoimmunthyreopathie: durch das (eigene) Immunsystem hervorgerufene Schilddrüsenerkrankung; [3]Biomarker: Messwert der Auskunft über einen bestimmten Zustand des Körpers gibt; [4]DNA: Träger der Erbinformation, [5]Basedow: eine Autoimmune Schilddrüsenerkrankung mit Schilddrüsenüberfunktion, [6]HRpQCT: hochauflösende CT-Untersuchung, die die Mikrostruktur des Knochens zeigt; [7]Genregulation: Veränderung der Menge an gebildeten Proteinen (=Eiweiße) [8]Zirkulierende miRNA: im Blutstrom befindliche miRNA

Einwilligungserklärung

Name der/s Probanden/in in Druckbuchstaben:

....

Geb.Datum: ..... Code: .....

Ich erkläre mich bereit, an der klinischen Studie ***"HashmiR"*** teilzunehmen.

Ich bin von Herrn/Frau……... ausführlich und verständlich über die klinische Studie, mögliche Belastungen und Risiken, sowie über Wesen, Bedeutung und Tragweite der klinischen Studie, sich für mich daraus ergebenden Anforderungen aufgeklärt worden. Ich habe darüber hinaus den Text dieser PatientInnenaufklärung und Einwilligungserklärung, die insgesamt 9 Seiten umfasst gelesen. Aufgetretene Fragen wurden mir vom Studienarzt/der Studienärztin verständlich und genügend beantwortet. Ich hatte ausreichend Zeit, mich zu entscheiden. Ich habe zurzeit keine weiteren Fragen mehr.

Ich werde den ärztlichen Anordnungen, die für die Durchführung der klinischen Studie erforderlich sind, Folge leisten, behalte mir jedoch das Recht vor, meine freiwillige Mitwirkung jederzeit zu beenden, ohne dass mir daraus Nachteile für meine weitere medizinische Betreuung entstehen.

Ich stimme ausdrücklich zu, dass meine im Rahmen dieser klinischen Studie erhobenen Daten wie im Abschnitt „Datenschutz“ dieses Dokuments beschrieben verwendet werden.

Für den Fall, dass ich aus der Studie ausscheide, bin ich einverstanden, dass meine Proben weiterhin aufbewahrt und analysiert werden, wie in dieser Information beschrieben: Ja ☐ Nein ☐

Mit der zusätzlichen Verwendung der pseudonymisierten Proben für Forschung und Lehre, die sich auf andere als die o.g. Studie bezieht, bin ich einverstanden.
Ja ☐ Nein ☐

.....
(Datum und Unterschrift der/s Patienten/in)

.....
(Datum, Name und Unterschrift des/der verantwortlichen Studienarztes/ärztin)

Eine Kopie dieser PatientInneninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt beim Studienarzt/der Studienärztin.

[1]miRNA-Signatur: Kombination verschiedener microRNAs (der DNA ähnelnde, kurze Moleküle, die die Herstellung von Genprodukten beeinflussen), [2]Immunthyreopathie/Autoimmunthyreopathie: durch das (eigene) Immunsystem hervorgerufene Schilddrüsenerkrankung; [3]Biomarker: Messwert der Auskunft über einen bestimmten Zustand des Körpers gibt; [4]DNA: Träger der Erbinformation, [5]Basedow: eine Autoimmune Schilddrüsenerkrankung mit Schilddrüsenüberfunktion, [6]HRpQCT: hochauflösende CT-Untersuchung, die die Mikrostruktur des Knochens zeigt; [7]Genregulation: Veränderung der Menge an gebildeten Proteinen (=Eiweiße) [8]Zirkulierende miRNA: im Blutstrom befindliche miRNA

ProbandInneninformation und Einwilligungserklärung zur Untersuchung des genetischen Hintergrundes im Rahmen der Beobachtungsstudie

Im Rahmen dieser Studie sollen genetische Varianten in folgenden für das Immunsystem wichtigen Genen untersucht werden:

- PTPN22 (Protein-Tyrosin Phosphatase-Gen) (rs2476601)
- DQB1*02 (MHC Klasse 2-Gen, Antigen-Präsentation für T-Zellen)
- DQA1*05 (MHC Klasse 2-Gen, Antigen-Präsentation für T-Zellen)
- DRB1*03 (MHC Klasse 2-Gen, Antigen-Präsentation für T-Zellen)

Auch die Untersuchung von [6]genregulatorischen Einflussfaktoren ist geplant. Dabei handelt es sich um Faktoren, die die Menge eines gebildeten Proteins ändern, ohne die Sequenz der DNA zu verändern (im Gegensatz zu genetischen Varianten). Dazu gehören im Blut vorkommende miRNAs.

<u>Folgende Maßnahmen zur Untersuchung von genetischen Parametern werden ausschließlich aus Studiengründen durchgeführt:</u>

Es werden bei jeder Ihrer ambulanten Untersuchungen 11 ml Blut (3 ml EDTA-Röhrchen und 8 ml Serumröhrchen) abgenommen. Das entspricht 1 Esslöffel Blut.

Aus der EDTA-Probe werden Blutzellen isoliert, DNA gewonnen und daraus verschiedene Varianten von Genen des Immunsystems (wie oben angeführt) untersucht.

Aus der Serumprobe wird weiteres Erbmaterial isoliert (RNA) und mittels spezieller Labormethoden die Art und Menge an zirkulierenden miRNAs untersucht.

Diese genetischen Untersuchungen werden zu wissenschaftlichen Zwecken durchgeführt. Eine Übermittlung der Gesamtergebnisse der Studie ist nach deren Abschluss gerne möglich, individuelle Ergebnisse können nur in Ausnahmefällen mitgeteilt werden.

[1]miRNA-Signatur: Kombination verschiedener microRNAs (der DNA ähnelnde, kurze Moleküle, die die Herstellung von Genprodukten beeinflussen), [2]Immunthyreopathie/Autoimmunthyreopathie: durch das (eigene) Immunsystem hervorgerufene Schilddrüsenerkrankung; [3]Biomarker: Messwert der Auskunft über einen bestimmten Zustand des Körpers gibt; [4]DNA: Träger der Erbinformation, [5]Basedow: eine Autoimmune Schilddrüsenerkrankung mit Schilddrüsenüberfunktion, [6]HRpQCT: hochauflösende CT-Untersuchung, die die Mikrostruktur des Knochens zeigt; [7]Genregulation: Veränderung der Menge an gebildeten Proteinen (=Eiweiße) [8]Zirkulierende miRNA: im Blutstrom befindliche miRNA

Einverständniserklärung für die genetische Untersuchung

Ich (Name der/s Probanden/in in Druckbuchstaben)

....

Geb.Datum:..... Code: .....

Ich bin von Herrn/Frau ..... über Wesen und Tragweite der genetischen Untersuchung aufgeklärt worden und erkläre mich bereit, an der genetischen Untersuchung im Rahmen der klinischen Studie **HashmiR** teilzunehmen.

Für den Fall, dass ich aus der Studie ausscheide, bin ich einverstanden, dass meine Proben weiterhin aufbewahrt und auf genetische Faktoren hin analysiert werden, wie in dieser Information beschrieben: Ja □ Nein □

.....
(Datum und Unterschrift der/des Patienten/in)

.....
(Datum, Name und Unterschrift des/der verantwortlichen Studienarztes/ärztin)

Eine Kopie dieser PatientInneninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt bei dem/der Studienarzt/ärztin

[1]miRNA-Signatur: Kombination verschiedener microRNAs (der DNA ähnelnde, kurze Moleküle, die die Herstellung von Genprodukten beeinflussen), [2]Immunthyreopathie/Autoimmunthyreopathie: durch das (eigene) Immunsystem hervorgerufene Schilddrüsenerkrankung; [3]Biomarker: Messwert der Auskunft über einen bestimmten Zustand des Körpers gibt; [4]DNA: Träger der Erbinformation, [5]Basedow: eine Autoimmune Schilddrüsenerkrankung mit Schilddrüsenüberfunktion, [6]HRpQCT: hochauflösende CT-Untersuchung, die die Mikrostruktur des Knochens zeigt; [7]Genregulation: Veränderung der Menge an gebildeten Proteinen (=Eiweiße) [8]Zirkulierende miRNA: im Blutstrom befindliche miRNA

PatientInneninformation[1] und Einwilligungserklärung zur Teilnahme an der Beobachtungsstudie

Graz Endokrinologie Register Studie

Sehr geehrte Teilnehmerin, sehr geehrter Teilnehmer!

Wir laden Sie ein an der oben genannten Beobachtungsstudie teilzunehmen. Die Aufklärung darüber erfolgt in einem ausführlichen Gespräch.

Ihre Teilnahme an dieser Studie erfolgt freiwillig. Sie können jederzeit ohne Angabe von Gründen aus der Studie ausscheiden. Die Ablehnung der Teilnahme oder ein vorzeitiges Ausscheiden aus dieser Studie hat keine nachteiligen Folgen für Ihre medizinische Betreuung.

Beobachtungsstudien sind Studien, bei denen in der Regel nur Daten aufgezeichnet und ausgewertet werden, die im Rahmen der normalen Patientenversorgung anfallen. In manchen Fällen kann es auch sein, dass zusätzliche, nicht belastende Untersuchungen oder Befragungen vorgenommen werden. In keinem Fall wird die für Sie vorgesehene Behandlung durch Ihre Studienteilnahme verändert. Beobachtungsstudien sind notwendig, um zusätzliche Erkenntnisse über bereits bewährte medizinische Verfahren zu gewinnen.

Zu dieser Beobachtungsstudie, sowie zur Patienteninformation und Einwilligungserklärung wurde von der zuständigen Ethikkommission eine befürwortende Stellungnahme abgegeben.

1. Was ist der Zweck dieser Studie?

Der Zweck dieser Beobachtungsstudie ist es Ihre medizinischen Daten die im Rahmen Ihrer Untersuchungen an der Ambulanz für Endokrinologie und Diabetologie erhoben werden in anonymer Form aufzuzeichnen um damit ein Registerdatenbank aufzubauen die dazu genützt werden sollte diverse wissenschaftliche Fragestellungen zu beantworten: z.B. den Zusammenhang ihrer Hormonwerte mit Ihren Beschwerden bzw. Ihrer eventuell vorliegenden Erkrankungen; die Registerdatenbank soll auch dazu dienen zu evaluieren wie gut die diagnostischen und therapeutischen Massnahmen an dieser Ambulanz funktionieren. Weiters haben wir durch Erhebung Ihrer Daten auch die Möglichkeit sie zu informieren falls es durch neue wissenschaftliche Untersuchungen Änderungen in der Therapie Ihrer Erkrankungen gibt, falls es Möglichkeiten zu Schulungskursen gibt oder es Möglichkeiten gibt an weiteren Studien teilzunehmen, von denen sie möglicherweise im Rahmen Ihrer Erkrankung profitieren könnten.

[1] Wegen der besseren Lesbarkeit wird im weiteren Text zum Teil auf die gleichzeitige Verwendung weiblicher und männlicher Personenbegriffe verzichtet. Gemeint und angesprochen sind – sofern zutreffend – immer beide Geschlechter.

2. Wie läuft die Beobachtungsstudie ab?

Diese Studie wird an der Ambulanz für Endokrinologie und Diabetologie durchgeführt, und es werden insgesamt *30.000* Personen daran teilnehmen.

Folgende Maßnahmen werden ausschließlich aus Studiengründen durchgeführt:

Es werden keinerlei Massnahmen aus Studiengründen bei Ihnen durchgeführt, sondern es werden lediglich ihre, im Rahmen der Routine sowieso erhobenen Daten in anonymisierter Form erfasst wobei wir sowohl die in der Vergangenheit, als auch die bei zukünftigen Ambulanzbesuchen erhobenen Daten erfassen werden.

3. Worin liegt der Nutzen einer Teilnahme an der Beobachtungsstudie?

Es ist nicht zu erwarten, dass Sie aus Ihrer Teilnahme an dieser Studie gesundheitlichen Nutzen ziehen werden, aber möglicherweise werden künftige Patienten mit der gleichen Erkrankung von den Ergebnissen profitieren.

4. Gibt es Risiken, Beschwerden und Begleiterscheinungen?

Nein.

5. In welcher Weise werden die im Rahmen dieser Beobachtungsstudie gesammelten Daten verwendet?

Sofern gesetzlich nicht etwas anderes vorgesehen ist, haben nur die Studienärzte und deren Mitarbeiter Zugang zu den vertraulichen Daten, in denen Sie namentlich genannt werden („personenbezogene" Daten). Weiters können ggf. Beauftragte von in- und ausländischen Gesundheitsbehörden, der zuständigen Ethikkommission und Personen, die vom Studienleiter und/oder Auftraggeber der Studie mit der Kontrolle der Datenqualität beauftragt wurden, Einsicht in diese Daten nehmen, um die Richtigkeit der Aufzeichnungen zu überprüfen. Diese Personen sind zur Verschwiegenheit verpflichtet.

Die Weitergabe der Daten erfolgt ausschließlich zu statistischen Zwecken und Sie werden ausnahmslos nicht namentlich genannt. Auch in etwaigen wissenschaftlichen Veröffentlichungen der Daten dieser Studie werden Sie nicht namentlich genannt.

Die Bestimmungen des Datenschutzgesetzes in der geltenden Fassung werden eingehalten.

6. Entstehen für die Teilnehmer Kosten? Gibt es einen Kostenersatz oder eine Vergütung?

Durch Ihre Teilnahme an dieser klinischen Prüfung entstehen für Sie keine zusätzlichen Kosten. Für Ihre Teilnahme an dieser klinischen Prüfung erhalten Sie keine Vergütung.

7. Möglichkeit zur Diskussion weiterer Fragen

Für weitere Fragen im Zusammenhang mit dieser Studie stehen Ihnen Ihr Studienarzt und seine Mitarbeiter gern zur Verfügung.

Name der Kontaktperson: Assoz. Prof. PD Dr. Stefan Pilz, PhD

An Wochentagen zwischen 8.00 und 16.00 regelmäßig erreichbar unter: 0316-385-81143

8. Einwilligungserklärung

Name des Patienten in Druckbuchstaben: .....

Geb.Datum: ..... Code: .....

Ich habe dieses Informationsblatt gelesen und verstanden. Alle meine Fragen wurden beantwortet und ich habe zurzeit keine weiteren Fragen mehr.

Mit meiner persönlich datierten Unterschrift gebe ich hiermit freiwillig mein Einverständnis, dass meine Daten gespeichert und ohne direkten Personenbezug für wissenschaftliche Zwecke verwendet werden dürfen. Mir ist bekannt, dass zur Überprüfung der Richtigkeit der Datenaufzeichnung Beauftragte der zuständigen Behörden und der Ethikkommission, sowie mit der Kontrolle der Datenqualität beauftragte Personen Einblick in meine personenbezogenen Krankheitsdaten nehmen dürfen.

Ich weiß, dass ich diese Zustimmungen jederzeit und ohne Angabe von Gründen widerrufen kann.

Eine Kopie dieser Patienteninformation und Einwilligungserklärung habe ich erhalten. Das Original verbleibt beim Studienarzt.

.....

(Datum und Unterschrift des Patienten)

.....

(Datum, Name und Unterschrift des verantwortlichen Arztes)

(Der Patient erhält eine unterschriebene Kopie der Patienteninformation und Einwilligungserklärung, das Original verbleibt im Studienordner des Studienarztes.)

genes

MDPI

Article

Expression Profiles of miR-22-5p and miR-142-3p Indicate Hashimoto's Disease and Are related to Thyroid Antibodies

Olivia Trummer [1,*,†], Ines Foessl [1,†], Natascha Schweighofer [1,2], Edi Arifi [1], Christoph W. Haudum [1,2], Sharmaine Reintar [1,2], Stefan Pilz [1], Verena Theiler-Schwetz [1], Christian Trummer [1], Andreas Zirlik [3], Albrecht Schmidt [3], Caterina Colantonio [3], Ewald Kolesnik [3], Nicolas Verheyen [3], Thomas R. Pieber [1,2] and Barbara Obermayer-Pietsch [1]

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† These authors contributed equally to this study.

check for updates

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Abstract: Hashimoto's thyroiditis (HT) is the most prevalent autoimmune disorder of the thyroid (AITD) and characterized by the presence of circulating autoantibodies evoked by a, to date, not fully understood dysregulation of the immune system. Autoreactive lymphocytes and inflammatory processes in the thyroid gland can impair or enhance thyroid hormone secretion. MicroRNAs (miRNAs) are small noncoding RNAs, which can play a pivotal role in immune functions and the development of autoimmunity. The aim of the present study was to evaluate whether the expression of 9 selected miRNAs related to immunological functions differ in patients with HT compared to healthy controls. MiRNA profiles were analysed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 24 patients with HT and 17 healthy controls. Systemic expressions of miR-21-5p, miR-22-3p, miR-22-5p, miR-142-3p, miR-146a-5p, miR-301-3p and miR-451 were significantly upregulated in patients with HT ($p \leq 0.01$) and were suitable to discriminate between HT and healthy controls in AUC analysis. Altered expressions of miR-22-5p and miR-142-3p were associated with higher levels of thyroid antibodies, suggesting their contribution to the pathogenesis of HT.

Keywords: miRNA; autoimmune thyroid disease; AITD; Hashimoto's thyroiditis

1. Introduction

Autoimmune thyroid diseases (AITDs) are the most common autoimmune diseases, affecting 2–5% of the population in high-income countries [1]. Hashimoto's thyroiditis (HT), the most frequent AITD, is the leading cause of hypothyroidism in iodine-sufficient areas of the world. Although exact mechanisms of aetiology and pathogenesis of HT are not completely understood, a strong genetic susceptibility to the disease has been confirmed by studies carried out within families and twins [2]. As in other autoimmune disorders, humoral and cellular immune mechanisms are closely related and cross-linked in AITDs. Disturbed self-tolerance accompanied by an increased antigen presentation is a precondition for their manifestation, based also on the interaction of thyroid, antigen presenting and T cells. Secreted cytokines provoke predominantly a T-helper type 1 (Th1)

as well as a Th17 response, which has been described [3]. Impaired thyroxin production and hypothyroidism as well as, more rarely, hyperthyroidism, are the consequences.

Early diagnosis and intervention may help to prevent the development of HT and abnormal thyroid function. The final diagnosis of HT depends on lymphocytic infiltration of the thyroid gland by fine-needle aspiration biopsy (FNAB) and further histopathological examination which is invasive and sometimes unfeasible [4]. Serum thyroid antibodies and ultrasonography are now used for diagnosis. At an early stage, HT is asymptomatic, easily leading to misdiagnosis [5]. Therefore, more biological markers need to be discovered to assist in early and accurate diagnosis of HT.

Micro RNAs (miRNAs) are small, noncoding, highly conserved ribonucleic acids (RNAs) that regulate gene expression by binding to messenger RNA (mRNA), thus modifying transcriptional processes. A single miRNA can regulate the expression of multiple genes and their encoded proteins [6]. In total, over 30% of human mRNAs are regulated by miRNAs [7]. Many miRNAs have been found to be important for the survival, development, differentiation, and function of T cells, B cells, dendritic cells, macrophages and other immune cell types [8,9]. Accordingly, differential miRNA expression profiles have been reported in autoimmunological disorders such as rheumatoid arthritis, systemic lupus erythematosus and psoriasis, [10–14] as well as in AITDs [15–19]. The aim of the study was to examine a panel of nine selected miRNAs to evaluate whether there is a difference in serum expressions of patients with HT and to investigate possible relations to thyroid antibodies. Candidate miRNAs for the present investigation have been selected according to their presence in serum as well as to previously described associations of humoral and/or cellular immune mechanisms involved in AITDs (Table 1).

Table 1. MiRNAs, mature sequence and source of reference of each selected miRNA.

micro RNAs	Sequence	Reference
hsa-miR-21-5p	5′UAGCUUAUCAGACUGAUGUUGA	[15]
hsa-miR-22-3p	5′AAGCUGCCAGUUGAAGAACUGU	[16]
hsa-miR-22-5p	5′AGUUCUUCAGUGGCAAGCUUUA	[16]
hsa-miR-96-5p	5′UUUGGCACUAGCACAUUUUUGCU	[15]
hsa-miR-142-3p	5′UGUAGUGUUUCCUACUUUAUGGA	[15]
hsa-miR-146a-5p	5′UGAGAACUGAAUUCCAUGGGUU	[15]
hsa-miR-301-3p	5′CAGUGCAAUAGUAUUGUCAAAGC	[15]
hsa-miR-375	5′UUUGUUCGUUCGGCUCGCGUGA	[16]
hsa-miR-451	5′AAACCGUUACCAUUACUGAGUU	[16]

hsa, homo sapiens; miRNA, micro RNA.

2. Materials and Methods

2.1. Study Populations

Data of the present investigation were obtained from the BioPersMed cohort ("Biomarkers of Personalized Medicine"), an ongoing single-centre, prospective, observational study to evaluate novel biomarkers for the assessment of cardiovascular and common metabolic diseases and their related complications. This observational trial was initiated in the year 2010 and the study population consists of 1022 asymptomatic subjects without diagnosed cardiovascular disease (CVD) with at least one classical risk factor for CVD, such as family history of CVD, hypertension or dyslipidaemia. Extensive anthropometric and clinical data were carefully recorded, including comorbidities such as previously diagnosed HT. Patients presenting with severe illnesses independent of aetiology, or who were expected not to be able to complete study specific examinations, have been excluded from participation. Moreover, persons with serious co-morbidities or mental health problems have also been excluded. Written informed consent from each participant was obtained after the study approval by the institutional review board of the Medical University of Graz (EC Nr. 24-224 ex 11-12). The BioPersMed study is conducted in compliance with Good Clinical Practice Guidelines Procedures (GCP) and carried out according to the principles of the Declaration of Helsinki.

For the present observational investigation, we screened the BioPersMed cohort for previously diagnosed HT patients (n = 27) as well as age and sex matched participants suitable as healthy controls (n = 22). HT patients have been diagnosed based on the commonly used diagnostic tools such as clinical manifestations, ultrasound and measurement of thyroid stimulating hormone (TSH), free triiodothyronine (fT3), free thyroxine (fT4), thyroglobulin autoantibody (TgAb) and thyroid peroxidase autoantibody (TPOAb) by their general practitioner or any other medical facilities. Exclusion criteria for HT patients were comorbidities such as acute (e.g., pancreatitis) or chronic inflammations (e.g., rheumatoid arthritis, polymyalgia, diabetes mellitus), endocrine disturbances in need of treatment (other than HT), history of myocardial infarction as well as history of cancers (e.g., bladder cancer, acoustic neuroma). Participants in the healthy control group showed at least one classical risk factor for CVD, but no serious comorbidities after a clinical validation by an experienced clinician. Serum samples were excluded if haemolysis was visually detected. We therefore excluded 3 samples of the HT group and 5 samples of the control group. In total, we investigated 24 HT patients compared to 17 healthy controls. A study flow chart is given in Figure 1.

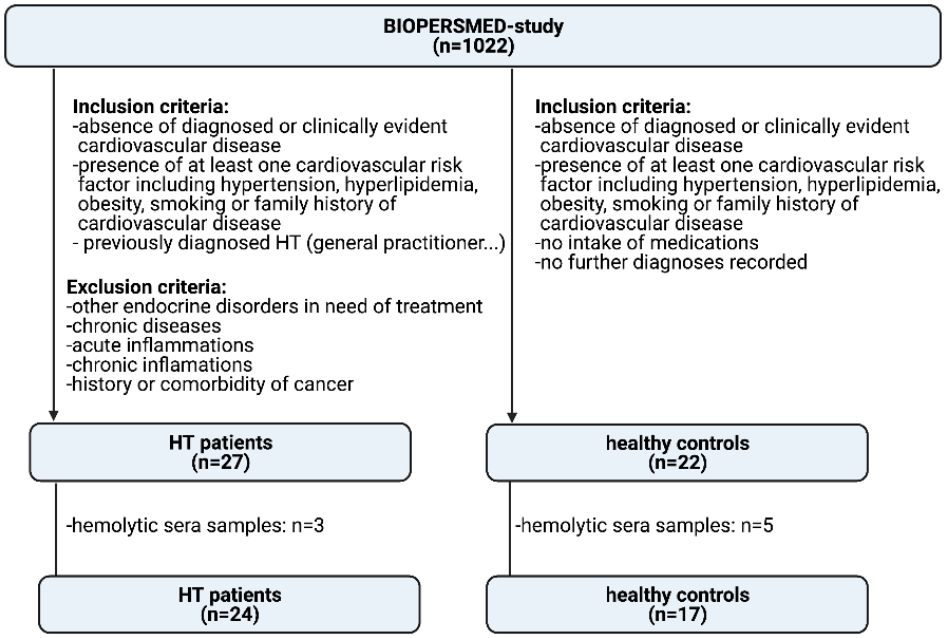


Figure 1. Study flow chart.

2.2. Patient Visit

Anthropometric data were measured in all participants. Baseline blood samples for laboratory analyses were collected between 7.00 and 9.00 a.m. after an overnight fast. Biobanking of blood samples was performed by freezing and storing the samples at −80 °C until analysis. To evaluate thyroid function and common autoantibodies, serum levels of TSH, fT3, fT4, TPOAb and TgAb were determined by luminescence immunoassay (Siemens, Erlangen, Germany) with intra- and inter-assay coefficients of variation (CV) of: TSH, 5.0% and 6.0%; FT3, 2.4% and 2.9%; FT4, 2.2% and 2.3%; TPO Ab, 5.2% and 6.1%, as well as Tg Ab, 5.0% and 4.6%, respectively. Body mass index (BMI) was calculated as body weight in kilograms (kg) divided by height in meters squared (m^2).

2.3. Selection of miRNAs

Based on previous studies [15,16], we selected 9 miRNAs that have been related to relevant immunological functions as candidates for the present investigation. These miRNAs are listed in Table 1.

2.4. miRNA Isolation and qPCR

MiRNA was isolated using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was eluted from the columns by addition of 20 µL RNase-free water, followed by centrifugation. The isolated miRNAs were short-term stored at −80 °C. Complementary DNA (cDNA) was generated using miRCURY LNA RT synthesis kit (Qiagen, Hilden, Germany), and subsequent quantitative real-time PCR (qPCR) was performed in duplicates using miRCURY LNA SYBR Green PCR Kit and specific miRCURY LNA miRNA PCR Assays (both from Qiagen, Hilden, Germany) with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Exogenous oligonucleotides have been added as spike-in controls (UniSp2, UniSp4, UniSp5, UniSp6 and cel-miR-39-3p) and were used to estimate the efficiency of RNA extraction, reverse transcription reaction and qPCR amplification (RNA Spike in Kit for RT, Qiagen, Hilden, Germany). All qPCRs were performed with interplate calibration, a maximum of 40 cycles were performed in duplicates and the average of cycle threshold (Ct) values were calculated. Only those miRNAs with a Ct < 37 were considered for further analysis. The relative expression levels of all investigated miRNAs were calculated as fold change [20]. For that, average Ct values have been normalized to spike-in controls to calculate ΔCt values. Fold change was calculated as $2^{-\Delta\Delta Ct}$ where ΔΔCt was ΔCt of HT patients minus ΔCt of controls. Quantitative qPCR data are reported as mean ± standard deviation (SD).

2.5. Functional Annotation of miRNAs

MiRWalk was used to identify potential target genes of differentially expressed miRNAs [21]. Matched binding sites have been evaluated in genes reportedly involved in the development of AITDs [22].

2.6. Statistical Analysis

Statistical analysis was performed using SPSS statistics version 25.0 (IBM SPSS Statistics GmbH, Ehringen, Germany). Patient characteristics and biomarker results are reported as mean ± SD unless otherwise stated. Distribution of data was analysed by descriptive statistics and Kolmogorov–Smirnov test, as well as by evaluation of quantile-quantile plots. Normally distributed quantitative data were compared using unpaired Student's *t*-test and unequally distributed data by applying Kruskal–Wallis tests for non-parametric samples. Changes of miRNA in the HT group are displayed as relative change compared to miRNA levels of healthy controls as reference. The diagnostic value for discriminating between HT patients and the control group was assessed by calculating the area under the curve (AUC). Receiver-operator characteristic (ROC) curves were generated by plotting sensitivity vs. (1-specificity). A *p*-value of ≤ 0.05 was considered as statistically significant. Adjustment for multiple testing has been performed by Bonferroni correction.

3. Results

3.1. General Results

We included a total of 41 participants, 33 women (81%), and 8 men (19%) in our analysis. Of these, 24 subjects (59%) were patients with previously diagnosed HT (22 women (92%) and 2 men (8%)). 17 subjects (41%) were classified as healthy (11 women (65%) and 6 men (35%)) (Figure 1).

In the HT patients group, 13% (n = 3) showed TgAb > 60 U/mL and 54% (n = 13) showed TPOAb > 60 U/mL. In total, 4 patients had both TgAb as well as TPOAb > 60 U/mL, respectively.

Of the HT patients, 29% (n = 7) were treated with levothyroxine. Control group participants did not take any recorded medication. Demographic data of the study population are given in Table 2.

Table 2. Demographic data of patients with Hashimoto's thyroiditis and healthy subjects. Frequency data are presented as number, (percentage), continuous data as mean ± standard deviation. HT, Hashimoto's thyroiditis; n, number; BMI, body mass index; FT3, free triiodothyronine; FT4, free thyroxine; TSH, Thyroid stimulating hormone; TPOAb, thyroid peroxidase autoantibody; TgAb, thyroglobulin autoantibody. Normal ranges of fT3, 3.0–6.3 pmol/L; fT4, 9.5–24 pmol/L; TSH, 0.10–4.0 µU/mL; TgAb; 0–60 IU/mL; TPOAb, 0–60 IU/mL.

	Patients with HT	Healthy Subjects
n	24	17
Sex female (*n*)	22 (91.7%)	11 (64.7%)
Age (yr)	57.9 ± 7.4	61.1 ± 5.8
BMI (kg/m^2)	25.2 ± 4.2	23.7 ± 2.34
fT3 (pmol/L)	4.4 ± 0.5	4.8 ± 0.4
fT4 (pmol/L)	15.9 ± 2.3	16.3 ± 2.0
TSH (µU/mL)	1.60 ± 0.87	1.83 ± 0.73
TgAb (IU/mL)	213.8 ± 365.6	
TPOAb (IU/mL)	212.2 ± 199.1	
Levothyroxine treatment	7 (29%)	

3.2. miRNA Expression Is Altered in Patients with HT

Systemic expression of miR-21-5p, miR-22-3p, miR-22-5p, miR-96-5p, miR-142-3p, miR-146a-5p, miR-301a-5p, and miR-451 was significantly upregulated in patients with HT. Associations of miR-21-5p, miR-22-3p, miR-142-3p, miR-146a-5p, miR-301-3p as well as miRNA-451 remained stable after Bonferroni correction. In contrast, miRNA-22-5p and miRNA-96-5p lost the level of significance after adjustment for multiple testing. Out of the nine selected miRNAs, miR-375 was the only candidate that was not upregulated in serum of HT patients. (Table 3). ΔCt values per group were normally distributed. Respective scatter plots are given in Figure 2. An annotation in miRWalk provided information on binding sites of the potential miRNAs.

Table 3. MiRNA ΔCt values according to the selected miRNAs of patients with Hashimoto's thyroiditis and healthy subjects. Data are shown as mean ± standard deviation.

hsa-miRNA	Patients with HT (*n* = 24)	Healthy Subjects (*n* = 17)	*p*-Value
miR-21-5p	−0.43 ± 0.54	0.68 ± 0.58	<0.001 *
miR-22-3p	1.58 ± 0.85	2.98 ± 0.80	<0.001 *
miR-22-5p	6.42 ± 0.95	7.22 ± 0.91	0.010
miR-96-5p	8.40 ± 1.21	9.22 ± 1.22	0.040
miR-142-3p	−0.84 ± 0.58	1.03 ± 0.60	<0.001 *
miR-146a-5p	2.71 ± 0.63	3.69 ± 0.65	<0.001 *
miR-301-3p	6.09 ± 1.01	7.21 ± 0.82	0.001 *
miR-375	8.52 ± 1.67	8.21 ± 1.10	0.503
miR-451	−2.60 ± 1.00	2.82 ± 1.35	<0.001 *

* indicates significant *p*-values after Bonferroni correction.

3.3. miR-22-5p and miR-142-3p Are Altered in HT Patients with Higher Levels of Thyroid Antibodies

Subgroup analyses within HT patients showed significantly higher miRNA expression for miR-22-5p in HT patients with higher thyroid antibody levels (TgAb and/or TPOAb > 60 U/mL, *n* = 13), 5.97 ± 0.74, as compared to HT patients with lower thyroid antibody levels (TgAb and/or TPOAb < 60 U/mL, *n* = 11) 6.95 ± 0.90; *p* = 0.008.

MiR-142-3p was also found to be significantly different (*p* = 0.05) in HT patients with higher levels of thyroid antibodies −0.25 ± 0.57 as compared to HT patients with thyroid antibody levels < 60 U/mL, 0.21 ± 0.51. In our regression analysis miR-22-5p and miR-142-3p expressions did not correlate with TPOAb levels (Figure 3).

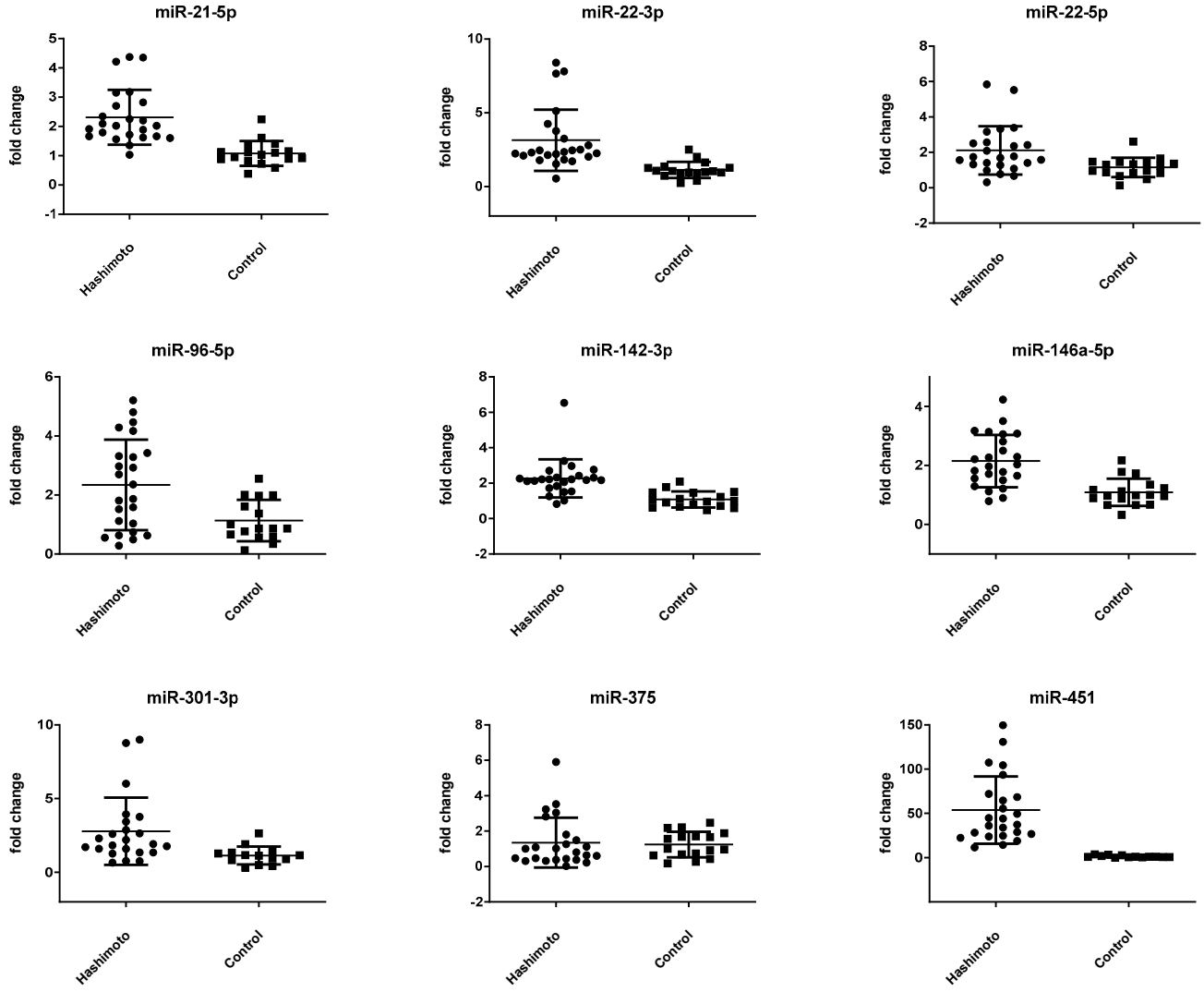


Figure 2. Expression of 9 miRNAs in serum of samples of HT patients and healthy controls. Data are displayed as scatter plots, where each dot represents the fold change as $2^{-\Delta\Delta ct}$-value of one study sample. Significance was tested by unpaired Student's *t*-test.

3.4. Potential Binding Site Targets

A functional annotation of these differentially expressed miRNAs revealed potential binding sites in genes of important immune mediators such as interleukins (IL), interferons (IFN), transforming growth factors (TGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). A scheme on how these miRNAs may interact in the development of AITD is given in Figure 4. Higher numbers of predicted miRNA binding sites were determined for miR-22-5p (n = 6) and for miR-142-3p (n = 4) compared to the mean number of binding sites for all miRNAs of 3.3 ± 2.0 (SD). MiR-21-5p showed potential interactions with IL-5, IFN-γ and IL-12. MiR-22-3p potentially interacts with IL-2, IL-5, IL-12, IL-17, IL-23, IFN-γ and TGF-β. MiR-22-5p potentially interacts with IL-1, IL-12, IL-13, IL-17 and IL-23 and IFN-γ. MiR-96-5p potentially interacts with IL-5, IL-13 and IL-23. MiR-142-3p potentially interacts with IL-1, TGFβ, IFN-γ and GM-CSF. MiR-146a-5p potentially interacts with IL-12, IL-5 and IL-17. MiR-301-3p potentially interacts with IL-7 and IL-17. MiR-451 potentially interacts with IL-1 and IL-12.

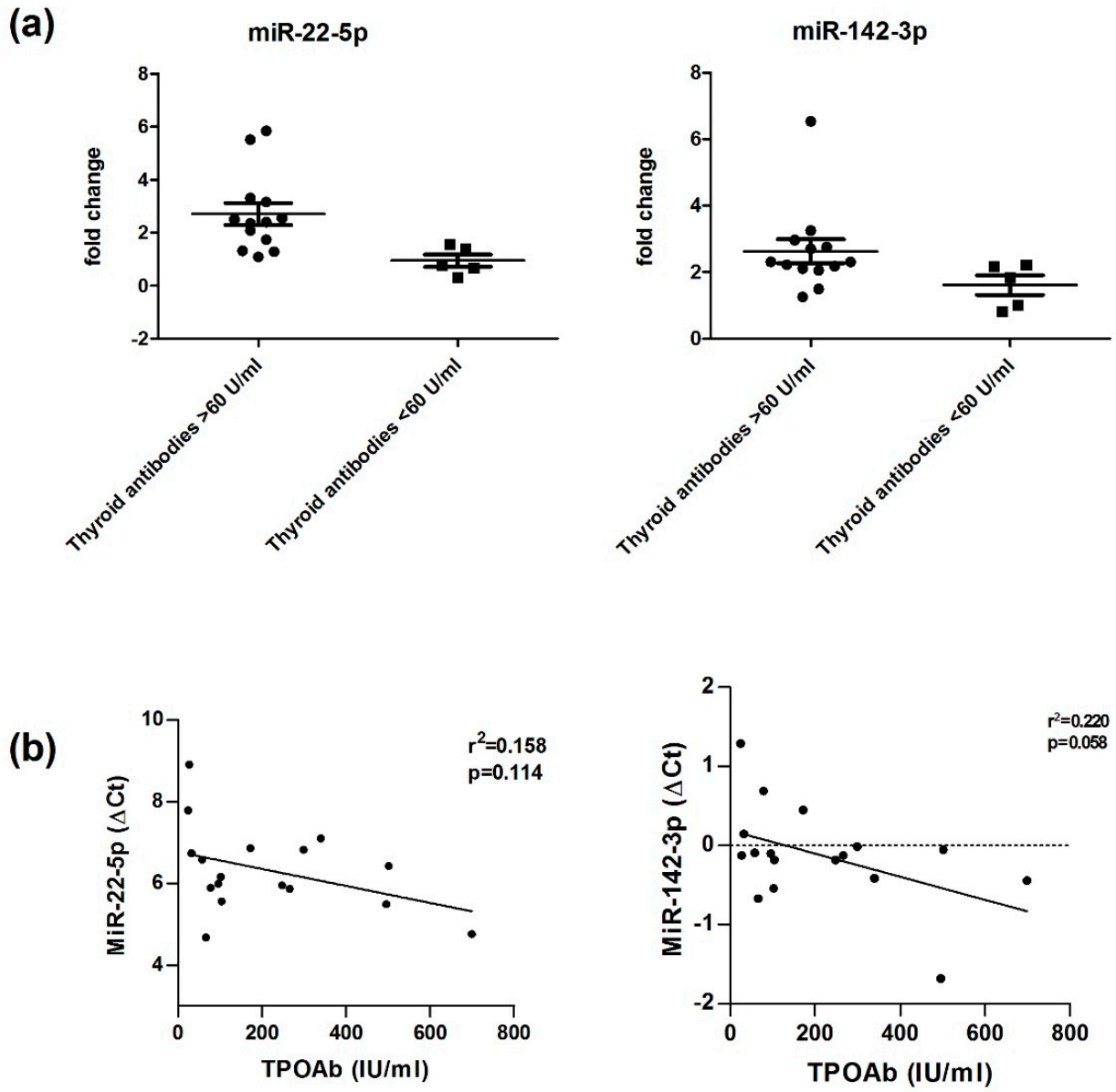


Figure 3. (**a**) Altered Expressions of miR-22-5p and miR-142-3p in serum of HT patients with higher levels of thyroid antibodies compared to HT patients with thyroid antibody levels <60 U/mL. Data are displayed as scatter plots, where each dot represents the ΔCt value of one HT patient. Significance was tested by unpaired Student's *t*-test. (**b**)Thyroid Antibodies (TPOAb) did not correlate with higher expressions of miR-22-5p and miR-142-3p (ΔCt values). TgAb, thyroglobulin autoantibody; TPOAb, thyroid peroxidase autoantibody; ΔCt, delta Cycle threshold.

3.5. miRNAs as Discriminators for HT Status in ROC Analysis

To evaluate the discriminatory potential of the differentially expressed miRNAs, we performed receiver-operating characteristic (ROC) analysis and calculated area under the curve (AUC) values. With the exception of miR-96-5p, differentially expressed miRNAs are "fair" (miRNA 22-5p: AUC = 0.76; 95% CI, 0.61–0.91; $p = 0.006$), "good" (miR-301-3p: AUC = 0.82; 95% CI, 0.68–0.96; $p = 0.001$ and miR-146a-5p: AUC = 0.86; 95% CI, 0.75–0.97; $p < 0.001$) or "excellent" (miR-21-5p: AUC = 0.99; 95% CI, 0.85–1; $p < 0.001$; miR-22-3p: AUC = 0.92; 95% CI, 0.82–1.00; $p < 0.001$; miR-142-3p: AUC = 0.92; 95% CI, 0.84–1.00; $p < 0.001$ and miR-451: AUC = 1.00; 95% CI, 1.00–1.00; $p < 0.001$) predictors [23] (Figure 5).

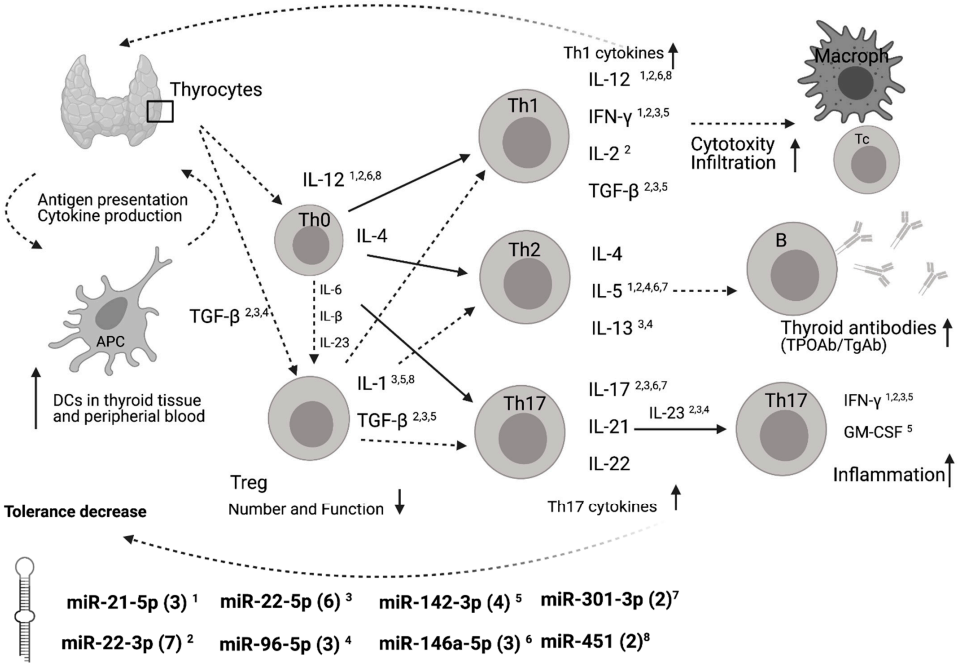
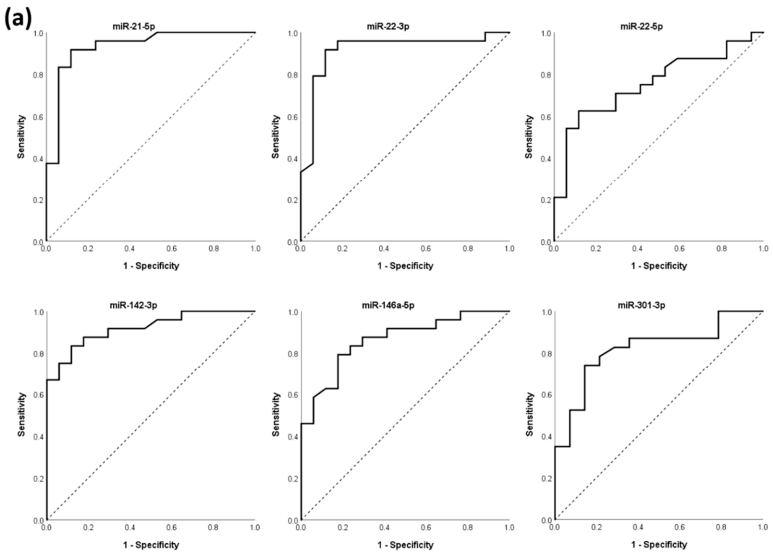


Figure 4. Summary of the main mechanisms related to autoimmunity of HT and potential interaction sites with differentially expressed miRNAs. Schematic representation of T cells differentiating into specific T cell subsets depending on the cytokines to which they are exposed and their main effects. MiRNA binding site predictions have been annotated by miRWalk database. Number of predicted binding sites are shown in brackets for each miRNA. Predicted binding sites of genes of HT immune-related molecules and/or their receptors are marked by superscript numbers. Adapted from [22]. APC, antigen presenting cell; Th, T helper cell; Macroph, macrophage; DC, dentritic cell; Treg, T-regulatory cells; TPOAb; peroxidase autoantibody; TgAb, thyroglobulin autoantibody, IL, interleukin; IFN-γ, interferon-γ; TGF-β, transforming growth factor β; GM-CSF, granulocyte-macrophage colony-stimulating factor; miR, miRNA.



miRNA	AUC	95%CI	*p*-value
miR-21-5p	0.93	0.85-1.00	<0.001
miR-22-3p	0.92	0.82-1.00	<0.001
miR-22-5p	0.76	0.61-0.91	0.006
miR-96-5p	0.68	0.51-0.85	0.055
miR-142-3p	0.92	0.84-1.00	<0.001
miR-146a-5p	0.86	0.75-0.97	<0.001
miR-301-3p	0.82	0.68-0.96	0.001
miR-375	0.45	0.27-0.63	0.560
miR-451	1.00	1.00-1.00	<0.001

Figure 5. The potential of differentially expressed miRNAs to discriminate between HT status and healthy controls, shown in ROC curves. (**a**) ROC curves of selected differently expressed miRNAs. (**b**) Calculated AUC values, 95% CI and *p*-values of each investigated miRNA. ROC, receiver-operating characteristic; DE, differentially expressed; AUC, area under the curve; CI, confidence interval.

4. Discussion

In the present observational study, eight out of nine observed miRNAs were differentially expressed in the serum of HT patients compared to healthy controls. In a subgroup analysis, HT patients with thyroid antibodies (TPOAb and/or TgAb) showed significantly higher expression levels for miR-22-5p and miR-142-3p but not for the other 7 miRNAs (Figure 3). To evaluate the accuracy of these differentially expressed miRNAs in predicting HT, we conducted a ROC analysis. AUC of these miRNAs were at least >0.76, indicating a "fair" test with enough balance between sensitivity and specificity for discriminating accurately between HT patients and healthy controls [24], (Figure 5).

The general tendency to overexpression detectable for all investigated miRNAs might be due to either active secretion as a consequence of increased inflammation in HT patients or derivation from autoimmune-related cell death. Independent from their cells of origin, miRNAs can function as endocrine signallers and are taken up by target cells [25].

We detected an increased expression of miR-22-5p and miRNA-142-3p, both associated with higher levels of serum antibodies (TgAb and/or TPOAb > 60 U/mL) in patients with HT. This corresponds with data of our ROC analysis. Both miRNAs are suitable to discriminate between HT patients and healthy controls. We could not confirm the association between antibody level group and altered expression by correlation analysis. Thyroid antibodies did not correlate with expression levels of miR-22-5p and miR-142-3p (Figure 3). Furthermore, and this counts for all of our analyses, we cannot estimate how the investigated miRNAs vary in their expressions before, during or after development of HT since this study was observational after development of HT.

Our results are in context with previously published data. Zhu et al. reported positive associations of TgAb levels and miR-142-5p but not for miR-142-3p [26], which is in line with our thyroid antibody analysis. Data of ROC curves are corresponding with ROC curves of Martínez-Hernández et al. with the exception of miR-96-5p. According to their analysis this miRNA is an "excellent" predictor (AUC = 0.91, 95% CI, 0.84–0.98) whereas our data failed the level of significance barely ($p = 0.055$) [24].

Our data of overexpression profiles in HT are in accordance with Martínez-Hernández et al. [15] as well as Yamada et al. [16], who both studied miRNA expressions in patients with AITD. The only non-concordant exception is miR-375, which was not differentially expressed in our cohort in contrast to Yamada's study. That in turn is in line with Zhao et al. who showed upregulated plasma levels of miR-375 and miR-451 in a four times larger study cohort than Yamada [5]. We are aware that caution must be taken when comparing miRNA data generated from different types of biofluids [27], since serum and plasma differ in their content of miRNA derived from different blood cells [28,29]. One of the potential reasons for the partly conflicting results of studies on miRNA expression in HT patients may be the interethnic expression differences between Asian (Yamada et al., Zhu et al.) and Caucasian (present study) cohorts [30]. In both investigations, serum miRNA profiling was performed by reverse transcription qPCR, the gold standard for sensitive and specific quantification of miRNAs in cell free biofluids. However, as frequently encountered, the quality of results varies in general strongly with the preanalytical steps such as blood drawing and serum/plasma preparation. The very low amounts of miRNAs, potentially high levels of inhibitors, biological variances of the individuals themselves (diet, exercise, age) as well as normalization strategies contribute to the variance in the results of different miRNA studies [31–33].

As confirmed by our in silico analysis, several T cell differentiation cytokines related to autoimmunity are potential targets of our overexpressed miRNA patterns (Figure 4).

MiR-22-3p binds 7 autoimmune-related cytokines, miR-22-5p has 6 binding partners and miR-142-3p has 4 binding partners. This may suggest a contribution of these miRNAs to differentiation of T cells into specific T cell subsets. MiR-22-5p regulates mainly cytokines involved in the differentiation of Th17 cells, promoting therefore the inflammatory response. Our focus was on miRNA targets related to autoimmunity of HT. We are aware that such

stringent criteria potentially excluded other regulatory interactions that might also play a role in the development of HT.

In our investigation miR-22-3p is associated with HT but not to serum thyroid antibodies. MiR-22-3p mainly targets genes of Th1 cytokines (IL-12, IFN-γ, IL-2 and TGF-β) but is binding partner of fewer TH2 cytokine genes (IL5) (Figure 4). This might suggest that miR-22-3p rather regulates the autoimmune related cytotoxicity and infiltration than changes in the development of thyroid antibodies. These theoretical assumptions are based on our in silico analysis. Whether cytokine levels are affected by the changed miRNA profile remains to be elucidated. This study was focussing on the biomarker aspect of the miRNA pattern. Nevertheless, our data showed serum overexpression of miR-22-5p and miR-142-3p related to the occurrence of thyroid antibodies.

Some further limitations of the study should be taken into account. There is still a debate on how and if normalization should be performed on qPCR results of serum miRNAs [34]. We decided to use exogenous controls (spike-ins) for normalization, excluding a potential bias by normalization on endogenous reference genes [33]. We cannot rule out a certain selection bias by choosing HT patients based on their previous medical history and not their prospective enrolment into the study. Further, cytokine levels were not determined in the BioPersMed cohort at the time of the patients' visits.

It should be kept in mind that published data, including the present study, lack long-term outcome data regarding disease activity and prognostic expectations. In this study, we present 2 miRNA candidates associated with higher occurrence of thyroid antibodies that possibly could be suitable to allow assumptions on whether HT patients are likely to develop higher titers of thyroid antibodies (TPOAb and or TgAb < 60 U/mL).

In conclusion, miRNA profiles of miR-21-5p, miR-22,3p, miR-22-5p, miR-142-3p, miR-146a-5p, miR-301-3p and miR-451 are upregulated in HT patients and suitable to discriminate between HT and healthy controls. Additionally, altered expressions of miR-22-5p and miR-142-3p are associated with higher levels of thyroid antibodies, suggesting important roles in the pathogenesis of HT.

Author Contributions: Conceptualization, O.T. and I.F.; methodology, O.T., I.F. and S.R. software, C.W.H.; validation, O.T. and I.F.; formal analysis, O.T.; investigation, S.P., V.T.-S. and C.T.; resources, B.O.-P. and T.R.P.; data curation, C.W.H.; writing—original draft preparation, O.T. and I.F.; writing—review and editing, I.F., A.Z., A.S. and C.C., visualization, E.K. and N.V.; supervision, B.O.-P.; project administration, E.A. and N.S.; funding acquisition, E.A. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Medical University of Graz (EC Nr. 24-224 ex 11-12).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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