

# microRNA Expressions in CD4<sup>+</sup> and CD8<sup>+</sup> T-cell Subsets in Autoimmune Thyroid Diseases

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## Key words

- micro-RNA
- autoimmune thyroid diseases
- Graves' disease
- Hashimoto's thyroiditis
- immunology

## Abstract

Graves' disease (GD) and Hashimoto's thyroiditis (HT) are the most common autoimmune thyroid diseases (AITD). MicroRNAs (miRNAs) critically control gene-expression and play an important role in regulating the immune response. The aim of this study was to prove significant variations of key immunoregulatory miRNAs in peripheral blood mononuclear cells (PBMCs) and in CD4<sup>+</sup> and CD 8<sup>+</sup> T-cells of AITD patients. Selected miRNAs were amplified by a semiquantitative SYBR Green PCR from PBMCs and purified CD4<sup>+</sup> and CD 8<sup>+</sup> T-cells of 59 patients with GD, HT, and healthy controls. Both GD and HT showed significantly decreased miRNA 200a<sub>1</sub> and miRNA 200a<sub>2</sub>\* in CD4<sup>+</sup>-T-cells (mean rela-

tive expression 12,57 in HT vs. 19.40 in control group (CG),  $p=0.0002$ ; 12,10 in GD vs. 19.40 in CG,  $p=0.0002$ ) and in CD8<sup>+</sup>-T-cells (13.13 in HT vs. 18,12 in CG,  $p=0.02$ ; 11.66 in GD vs. 18.12 in CG,  $p=0.0002$ ). GD and HT showed significantly decreased miRNA 155<sub>2</sub> and miRNA 155\*<sub>1</sub> in HT in CD8<sup>+</sup>-T-cells (10.69 in HT vs. 11.30 in CG,  $p=0.01$ ; 10.40 in GD vs. 11.30 in CG,  $p=0.005$ ). This study confirms significant variations of miRNA200a and miRNA155 in patients suffering from GD and HT in vivo in CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells. These data may help to better understand the gene regulations in the causative cells causing these autoimmune processes. They extend our very limited knowledge concerning miRNAs in thyroid diseases.

received 19.08.2013  
first decision 21.10.2013  
accepted 04.11.2013

## Bibliography

DOI <http://dx.doi.org/10.1055/s-0033-1361088>  
Exp Clin Endocrinol Diabetes 2014; 122: 107–112  
© J. A. Barth Verlag in Georg Thieme Verlag KG Stuttgart · New York  
ISSN 0947-7349

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## Introduction

### Autoimmune thyroid diseases

Graves' disease (GD) and Hashimoto's thyroiditis (HT) are the most common autoimmune thyroid diseases (AITD). Both AITDs are similar in terms of lymphocytic infiltrations of the thyroid gland. However, underlying cellular and humoral immune responses are clearly different in the 2 entities.

HT is characterized by infiltration of autoreactive T and B cells into the thyroid gland, causing thyroid cell death [1] and production of anti-thyropoxidase (TPO) and anti-thyroglobulin (Tg) antibodies [2]. We could previously show that cytotoxic CD8<sup>+</sup> T-cells of HT patients recognize TPO- and Tg-antigens [3] and in cooperation with natural killer cells are involved in the disease process of HT [4]. Regulatory CD4<sup>+</sup> T-cells are believed to play an important role in the moderation of this autoimmunological process in Hashimoto's thyroiditis [5,6].

The etiology of GD is dependent on the fact that thyroid auto-reactive T-cells escape immune

tolerance, infiltrate the thyroid gland and induce activation of auto-reactive B-cells that secrete thyrotropin receptor (TSH-R)-stimulating antibodies (TRAb) which cause hyperthyroidism [7]. In GD, both Th1 mediating cell mediated immunity and Th2 mediating humoral immunity occur [8]. Cytokine expression profiles in sera and thyroid tissues from Graves' disease patients indicate a mixed Th1/Th2 status at any time [9]. Through both immune responses complex interactions of cytokines and chemokines mediated also through CD4<sup>+</sup> T-cells are enabled, which beside inducing activation of auto-reactive B-cells, also lead to an activation of CD8<sup>+</sup> T-cells contributing to the toxic reaction in the thyroid tissue [10].

The current etiopathological dogma is that AITDs are complex diseases where on the basis of susceptibility genes, environmental triggers initiate the autoimmune response to the thyroid.

### microRNAs

microRNAs (miRNAs) constitute a family of small RNAs, 21–25-nucleotides in length, which control

gene expression by virtue of complementary binding to targeted mRNA-sequences and consecutive disposal of double-stranded RNAs [11]. Since the identification of the miRNA lin-4 as a regulator of developmental timing in the nematode *Caenorhabditis elegans* [12] it has become evident that these short non-coding RNAs act post-transcriptionally to regulate eukaryotic gene expression [13]. Accumulating data suggest that miRNA control is an important feature of the mammalian immune system. Genetic ablation of certain individual miRNAs machinery severely compromises immune development and regulation, implicating the influence of miRNAs in the pathophysiology of both immunity and autoimmunity [14, 15].

In adaptive immune responses, dynamic alterations in miRNA expression have been noted and were attributed to changes in gene expression of T-cells, relating to lineage commitment and stepwise maturation [16]. Several studies have reported the involvement of miRNAs in immune cell development [17–19]. These studies demonstrated that amongst others, miR-150, miR-155, miR-181a are important regulators of B and T cell development and play crucial roles for the establishment of a functional adaptive immune system [18, 19].

The discovery of several key proteins in the biogenesis of miRNAs, Droscha/DGCR8, Dicer, and Argonautes in mammals, have allowed the simultaneous non-selective or partially selective ablation of hundreds of miRNAs while leaving protein-coding genes intact [20,21]. Selective deletion of Dicer [22,23] and other key proteins in individual immune subsets has been used to demonstrate that miRNAs are critical for B, NK, NKT, and T-cell development, function, and lineage stability of terminally differentiated lymphocytes [24, 25].

In conclusion, it has become increasingly clear from cell culture and animal studies that proper miRNA regulation is critical for the prevention of autoimmunity and normal immune functions.

### role of miRNAs in AITDs

Recently levels of key immunoregulatory miRNAs in thyroid glands tissue of AITD patients and healthy controls have been determined by us [26]. We assumed that these miRNA variations are caused through infiltrating activated lymphocytes, because – according to additionally performed microscopy of fine needle aspirations – these cells are dominant. To best of our knowledge currently there is no more data concerning this important issue. The main aim of this study was to prove significant variations of the key immunoregulatory miRNAs 155\_2, miRNA 200a1, miRNA 146a1 and others selected after a careful review of the literature in PBMCs and in purified CD4+ and CD8+ cells T-cells of AITD patients and healthy controls.

## Material and Methods



### Ethics statement

The study protocol was approved by the local ethics committee: Ethikkommission der Medizinischen Fakultät der Heinrich-Heine Universität Duesseldorf Germany (no. 3354) and the investigations conform to the principles outlined in the Declaration of Helsinki. All participants gave written informed consent before enrolment.

### Study design and participants

This study aimed at investigating the levels of selected microRNAs in PBMCs and in CD4+ and CD8+ T-cells of patients with

Hashimoto thyroiditis (HT) and Graves's disease (GD). The chosen miRNAs were selected after a careful review of the literature including GWA analysis. For TPO antibodies measurement we utilized a commercial assay using a 2-step chemiluminescence sandwich assay using directly coated magnetic microparticles (DiaSorin Kitinsert Liaison® Anti-TPO) with a sensitivity of 51.5% and a specificity of 95% determining HT. Based on the low sensitivity we defined HT by an at least 3 fold increase of TPO antibodies over the maximum normal range in serum and additionally a typical diffuse hypoechogenic pattern on ultrasound. TRAb measurement was performed by a commercial, porcine radioreceptorassay (RRA) (DiaSorin Kitinsert Liaison® TRAbs) with a sensitivity of 98.3% and a specificity of 99.2% determining GD. Although sensitivity and specificity were high we decided in concordance with the criteria for HT to define GD by an at least 3 fold increase of TSH-receptor antibodies and additionally a hypoechogenic hypervascularized thyroid parenchyma on ultrasound. The control group consisted of age-matched individuals without autoimmune diseases. In all groups patients with a history of any neoplasm including thyroid carcinoma or currently treated for any neoplasia were excluded. Furthermore, in all participants, acute or chronic infections were excluded by clinical examination, measurement of C-reactive protein and total blood count. Baseline data included blood levels of thyroid antibodies, hormone levels of fT3, fT4 and TSH. No study participant was affected by another autoimmune process.

At the time of taking blood samples, all GD patients were under thyreostatic therapy with methimazole or carbimazole. The treatment duration with methimazole or carbimazole lasted from 1 week to 6 months. 13 out of 19 patients were hyperthyroid, 6 were euthyroid. 18 of 21 HT patients were treated with L-Thyroxine in varying dosages (50µg–100µg once daily). The treatment duration with L-Thyroxine in HT patients lasted from at least 6 months up to 12 years. All of the HT patients were euthyroid. All 19 patients in the control group were euthyroid. Blood samples were drawn from an antecubital vein with subjects in supine position to PAXgene blood tubes (PreAnalytix) and to heparinized BD Vacutainer Cell Preparation Tubes (BD Biosciences). PAXgene blood tubes were incubated at room temperature for at minimum 4h before freezing for further purpose by –80°C. Peripheral blood mononuclear cells (PBMC) were isolated by centrifuging (3100 RPM) heparinized whole blood samples in BD Vacutainer Cell Preparation Tubes and PBMC were frozen immediately after centrifugation for further purpose by –80°C.

### Semiquantitative SYBR Green PCR

Total RNA, including miRNA, was extracted from blood samples collected in PAXgene tubes with Pax Gene Blood microRNA Kit (PreAnalytiX) according to manufacturer's instructions. CD4 and CD8-positive T-cells were isolated from PBMC won out of BD Vacutainer Cell Preparation Tubes by magnetic bead separation and resuspended in 0.7 ml Qiazol Lysis Reagent (Qiagen) using first the Pan T-cell Separation Kit (Milteny Assay) and afterwards by CD4/CD8-positive T Micro Beads (Milteny Assay). Afterwards total RNA, including miRNA, was extracted from CD4+ and CD8+ cells and reversely transcribed with miRNeasy Mini Kit (Qiagen GmbH, Germany). All procedures were accomplished as described in the manufacturing protocols.

The total RNA concentration was quantified in all samples using NanoDrop ND-1000 Spectrophotometer (peqlab Biotechnologie GmbH, Germany) and the cell samples were stored at –80°C

until further use. The same amount of miRNA was transcribed to cDNA for every sample with miScript II RT Kit (Qiagen GmbH, Germany) according to the kit protocol. Semiquantitative SYBR Green PCR was performed in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, USA) using the miScript SYBR Green PCR Kit (Qiagen GmbH, Germany). The specific miScript Primer Assays (Qiagen GmbH, Germany) were used for the amplification of miRNA 34a (no sequence listed), 143\_1 (5'UGA-GAUGAAGCACUGUAGCUC), 146a\_1 (5'UGAGAACUGAAUUC-CAUGGGUU), 155\*\_1 (5'CUCUACAUUUAGCAUUAACA), 155\_2 (no sequence listed), 181a\*\_1 (5'ACCAUCGACCGUUGAUU-GUACC), 181b1 (no sequence listed), 200a1 (no sequence listed), 200a\*2 (no sequence listed) The samples were incubated in a 96-well optical plate at 95 °C for 15 min as initial activation step, followed by 40 cycles of a 3-step cycling: 94 °C for 15 s, 55 °C for 30 s, 70 °C for 30 s. Each sample was checked in duplicate. For negative control reactions, containing only RNase free water, the reverse transcription step was omitted. RNU6b was used as an endogenous control.

The miRNA of interest were relatively quantified in relation to endogenous control gene using the comparative cycling threshold (Ct) method in separate tubes.

### FACS-analysis

We verified in 3 control patients CD4 and CD8-positive T-cell isolation by magnetic bead separation. Therefore we primary performed a HLA typing by flow cytometry as described before [27]. Afterwards CD4+ and CD8+ T-cells isolation was conducted according to manufacturer's instructions. Finally the purity of this separation was evaluated by fluorescence-activated cell sorting (FACS) analysis as described before [27]. In all controls the purification reached over 90%.

### Statistics

All data were analyzed with the Statistical Package for the Social Sciences 20.0 (2011). Due to small sample sizes, differences between the mean relative miRNA expression of the control group and HT as well as GD patients were compared by means of the non-parametric Kruskal-Wallis-test. Results with  $p < 0.05$  were considered statistically significant. Bonferroni-corrected

Mann-Whitney U-tests were used as posthoc tests in case the overall Kruskal-Wallis-test was significant, i.e., the conventional critical  $\alpha$ -level of 0.05 was divided by the number of posthoc analyses ( $0.05/2=0.025$ ) with  $p < 0.025$  indicating significant posthoc differences.  $\chi^2$  (Fisher's exact) tests were used to assess differences between proportions. In  $\chi^2$ -tests,  $p < 0.05$  indicated statistical significance.

### Results

Altogether  $n=59$  unrelated Caucasians were included in this study: The control group comprised 19 Caucasians without AITD [ $n=19$ ; mean age, 42.26 years (range, 22–79 years), 12 females], GD group [ $n=19$ ; mean age, 48 years (range, 20–83 years), 13 females], HT group [ $n=21$ ; mean age, 40 years (range, 12–67 years), 19 females]. A Kruskal-Wallis test showed no significant group differences with regard to patients' mean age,  $p=0.289$ . We investigated miRNA 34a\_1, miRNA 143\_1, miRNA 146a\_1, miRNA 155 with the 2 maturity sequences 155\*\_1 and 155\_2, miRNA 181a\*\_1, miRNA 181b\_1, miRNA 100a with the 2 maturity sequences 200a\_1 and 200a\_2\*.

At first we analyzed the miRNA levels in PBMCs in 19 GD patients, 21 HT patients and 19 controls by semiquantitative SYBR Green PCR. We found that miRNA 146a\_1 is statistically significantly increased in PBMCs of GD patients vs. controls: We found mean relative expression 9.42 in GD group vs. 6.87 in control group,  $p=0.017$  (Table 1).

In a further step we analyzed the miRNA levels in the CD4+ T-cells in the peripheral blood in 10 GD patients, 10 HT patients and 10 controls by semiquantitative SYBR Green PCR. We found statistically significant changes in expression levels of several miRNAs compared to controls (Table 2): miRNA 200a\_1 (mean relative expression 12.57 in HT group vs. 19.40 in control group,  $p=0.0002$ ) and miRNA 200a2\* (12.62 in HT group vs. 17.94 in control group,  $p=0.0004$ ) are significantly decreased in CD4+ T-cells of HT patients vs. controls. We found further that miRNA 200a\_1 (12.1 in GD group vs. 19.40 in control group,  $p=0.0002$ ) and miRNA 200a2\* (13.37 in GD group vs. 17.94 in control

**Table 1** Mean relative miRNA expression in PBMCs by group.

miRNA of PBMCs No. of patients	Control 19		GD 19		HT 21		Kruskal-Wallis Test p-value
	Mean	SD	Mean	SD	Mean	SD	
34a_1	14.9845	2.51723	13.5299	1.46940	13.5659	3.14225	n. s.
143_1	15.9642	3.40512	15.8082	1.26454	16.2047	2.75941	n. s.
<b>146a_1</b>	<b>6.8714</b>	3.82770	<b>9.4228</b>	2.29542	9.0465	3.56513	<b>0.030</b>
<b>155*_1</b>	<b>17.4560</b>	4.77092	<b>18.4689</b>	0.90446	16.6119	2.26680	<b>0.021</b>
155_2	11.0771	1.47403	11.5542	1.58796	11.8492	1.40678	n. s.
181a*_1	14.8500	4.22941	15.4677	2.95393	15.6860	3.09205	n. s.
181b_1	8.6005	2.91385	9.4407	2.36097	8.3295	2.49579	n. s.
200a_1	15.1491	3.33402	15.4816	5.62126	15.6612	5.29238	n. s.
200a_2*	10.1294	7.00531	13.4610	2.82750	14.2450	2.76764	n. s.

Means in bold differ significantly. SD = standard deviation. n. s. = non significant

Bonferroni-corrected Mann-Whitney-U test for post hoc analysis.

miRNA of PBMCs	Control vs. HT p-value	Control vs. GD p-value	n. s.
<b>146a_1</b>	0.027	<b>0.017</b>	<b>GD &gt; Control</b>
155*_1	0.047	0.872	

Values in bold are significant at a Bonferroni-corrected  $p$ -level of 0.025

**Table 2** Mean relative miRNA expression in CD4+ T-cells by group.

miRNA of CD4+ No. of patients	Control		GD		HT		Kruskal-Wallis Test p-value
	10		10		10		
	Mean	SD	Mean	SD	Mean	SD	
34a_1	12.6734	1.8820	13.8120	2.4372	12.0646	4.1020	n.s
143_1	18.9467	1.0710	18.2813	2.4098	16.6490	4.2364	n.s
146a_1	7.8378	0.4216	8.1385	1.8992	5.8541	3.1389	n.s
<b>155*_1</b>	<b>15.9214</b>	1.2005	16.6349	1.5145	<b>13.5349</b>	3.2657	<b>0.0276</b>
155_2	10.5570	0.4059	10.6363	2.1738	10.0531	2.2689	n.s
181a*_1	11.9881	4.4635	16.9229	2.1391	14.9553	4.8634	n.s
181b_1	9.6901	0.5363	10.3465	2.1807	8.5635	3.8393	n.s
<b>200a_1</b>	<b>19.4026</b>	0.8500	<b>12.1005</b>	0.8669	<b>12.5701</b>	4.8645	<b>0.0001</b>
<b>200a_2*</b>	<b>17.9417</b>	2.2590	<b>13.3650</b>	0.88267	<b>12.6208</b>	1.6726	<b>0.0003</b>

Means in bold differ significantly. SD=standard deviation. n.s.=non significant

Bonferroni-corrected Mann-Whitney-U test for post hoc analysis.

miRNA of PBMCs	Control vs. HT p-value	Control vs. GD p-value	
155*_1	0.04937	0.70546	n.s.
<b>200a_1</b>	<b>0.00021</b>	<b>0.00016</b>	<b>Control&gt;GD/HT</b>
<b>200a_2*</b>	<b>0.00038</b>	<b>0.00088</b>	<b>Control&gt;GD/HT</b>

Values in bold are significant at a Bonferroni-corrected p-level of 0.025

**Table 3.** Mean relative miRNA expression in CD8+ T-cells by group.

miRNA of CD8+ No. of patients	Control		GD		HT		Kruskal-Wallis Test p-value
	10		10		10		
	Mean	SD	Mean	SD	Mean	SD	
miRNA 34a_1	12.7840	1.7684	13.3987	2.0206	12.6489	3.6591	n.s.
miRNA 143_1	17.3819	3.3645	17.6205	2.5990	16.7469	3.9380	n.s.
miRNA 146a_1	8.7585	1.3284	7.9438	1.9700	6.3416	3.2809	n.s.
<b>miRNA 155*_1</b>	<b>17.0667</b>	1.0473	16.3847	1.5057	<b>14.2522</b>	2.4194	<b>0.0040</b>
<b>miRNA 155_2</b>	<b>11.3007</b>	0.7141	<b>10.3945</b>	1.8811	<b>10.6874</b>	1.8109	<b>0.0069</b>
miRNA 181a*_1	13.2699	4.3064	16.3354	2.0049	14.6384	4.9741	n.s.
miRNA 181b_1	10.3096	1.8967	9.4823	1.9816	8.6013	3.4773	n.s.
<b>miRNA 200a_1</b>	<b>18.1190</b>	2.7510	<b>11.6572</b>	0.6286	<b>13.1275</b>	4.3749	<b>0.0011</b>
<b>miRNA 200a_2*</b>	<b>17.8437</b>	2.4541	<b>12.5372</b>	1.0043	<b>13.1028</b>	1.3013	<b>0.0002</b>

Means in bold differ significantly. SD=standard deviation. n.s.=non significant

Bonferroni-corrected Mann-Whitney-U test for post hoc analysis.

miRNA of PBMCs	Control vs. HT p-value	Control vs. GD p-value	
<b>155*_1</b>	<b>0.00650</b>	<b>0.05878</b>	<b>Control&gt;HT</b>
<b>155_2</b>	<b>0.01017</b>	<b>0.00516</b>	<b>Control&gt;GD/HT</b>
<b>200a_1</b>	<b>0.01911</b>	<b>0.00016</b>	<b>Control&gt;GD/HT</b>
<b>200a_2*</b>	<b>0.00067</b>	<b>0.00051</b>	<b>Control&gt;GD/HT</b>

Values in bold are significant at a Bonferroni-corrected p-level of 0.025

group,  $p=0.0009$ ) are also significantly decreased in CD4+ T-cells of GD patients vs. controls.

Further on we looked after the miRNA levels in the CD8+ T-cells in the peripheral blood in the same 10 GD patients, 10 HT patients and 10 controls by semiquantitative SYBR Green PCR (Table 3). In HT patients we found significantly decreased miRNA 155\*\_1 (14.25 in HT group vs. 17.07 in control group,  $p=0.007$ ) and miRNA 155\_2 (10.69 in HT group vs. 11.30 in control group,  $p=0.01$ ). miRNA 200a\_1 (13.13 in HT group vs. 18.12 in control group,  $p=0.02$ ) and miRNA 200a\_2\* (13.10 in HT group vs. 17.84 in control group,  $p=0.007$ ) we found also statistically significantly decreased compared to controls. In GD patients we detected statistically significant decrease of miRNA 155\_2 (10.40 in GD group vs. 11.30 in control group,  $p=0.005$ ), miRNA 200a\_1 (11.66 in GD group vs. 18.12 in control group,  $p=0.0002$ ) and

miRNA 200a\_2\* (12.54 in GD group vs. 17.84 in control group,  $p=0.0005$ ) when compared to controls. No significant change was found in GD for miRNA 155\*\_1.

## Discussion

The exact etiology of the immune response in GD and HT to the thyroid is still unknown. The present study was undertaken to further evaluate the influence of defined miRNAs under suspicion to be involved in immune regulation in the thyroid tissue of patients suffering from AITDs.

We demonstrate that the miRNA 146a\_1 is significantly increased in PBMC of GD patients as compared to controls. Analysis of miRNA-146a gene expression unveiled a pattern of induc-

tion in response to a variety of microbial components and proinflammatory cytokines [28]. In human lung alveolar epithelial cells, for example, increased miRNA-146a expression was found to negatively regulate the release of the proinflammatory chemokines IL-8 and RANTES [29,30]. Concerning autoimmune diseases, Nakasa et al. reported that miRNA-146a was highly expressed in rheumatoid arthritis (RA) synovial tissue compared to osteoarthritis and normal synovial tissue [31]. Our previous study could show for the first time a significant decrease of miRNA-155\_2 in the thyroid tissue of HT patients [26]. We could now demonstrate that miRNA-146a is statistically significantly increased in PBMCs of GD patients in accordance to Nakasa et al. findings [31]. This result underlines the idea that GD is crucially caused by emigrating mononuclear cells producing TH2 cytokines. Through our data we can raise the thesis that modified PBMCs with increased miRNA-146a infiltrate the thyroid tissue of GD patients and may be critically involved in composing the local cytokine milieu in GD. In their meta-analysis Chen et al. could not find an association between miR-146a G/C rs2910164 polymorphism and the development of autoimmune diseases [32]. That might imply that autoimmune diseases are caused by an increase of miRNA146a and not by a miRNA146a polymorphism.

Furthermore we could show that both sequences of miRNA 155 (miRNA 155\*\_1 and miRNA 155\_2) are significantly decreased in CD8+ T-cells of HT patients vs. controls and that the sequence miRNA 155\_2 is also significantly decreased in CD8+ T-cells of GD patients vs. controls. Concerning miR-155, Thai et al. have shown that miRNA-155 has an important role in the mammalian immune system by specifically regulating T-helper cell differentiation and the germinal center reaction to produce an optimal T cell-dependent antibody response [33]. Because of his spadework, we considered miRNA-155 a suitable candidate miRNA modulating and being modulated in AITDs. Our previous study could show for the first time a significant decrease of miRNA-155\_2 in the thyroid tissue of HT patients [26]. In the present work we could demonstrate that miRNA 155\* 1 and miRNA 155\_2 are significantly decreased in CD8+ T-cells in the peripheral blood of HT patients. As shown previously in HT, CD8+ T-cells become activated, and infiltrate into the thyroid [3]. We believe that the genetically modified CD8+ positive T-cells infiltrate the thyroid. We conclude that the post-transcriptional regulation caused through the decrease of miRNA 155 in cytotoxic CD8+ T-cells might contribute to the pathological identification of the thyroid specific cellular antigens TPO and Tg through the CD8+ T cells and therefore being involved in the thyroid destruction as described previously. These ideas, of course, need further research.

We also found a decrease of miRNA155\_2 in cytotoxic CD8+ T-cells in GD patients in peripheral blood. Although it becomes increasingly clear that CD8+ T-cells activated by autoreactive B-cells contribute to the toxic reaction in the thyroid tissue in GD patients [10] up to now, the exact mechanism of this activation remains uncertain. It is believed that the activation is caused through a tricky interaction of chemokines and cytokines. In the context, the present results prompt an interesting, testable hypothesis: May the identified chemo- and cytokines in GD influence the level of miRNA 155 in GD, that consecutively cause post-transcriptional regulations of CD8+ T-cells?

Recent large-scale genome-wide association (GWA) studies of single nucleotide polymorphism (SNP) variations captured many thousands individual genetic profiles of *H. sapiens* and

facilitated identification of significant genetic traits which are highly likely to influence the pathogenesis of several major human diseases. Glinsky analysed for the first time gene expression patterns of miRNAs in association to AITDs [34,35]. He could show that several miRNAs including miRNA200 were associated with AITD in general and GD in particular. Prior to this finding the miRNA200 family has only been tied to the development and proliferation of various types of cancer [36,37]. In the current study we found a decrease of miRNA 200a\_1 and miRNA 200a2\* in CD4+ and CD8+ T-cells in peripheral blood but not in PBMCs of GD and HT patients. We believe that these contradictory findings in peripheral blood cells and thyroid tissue might be caused through involvement of different cells. A decrease of miRNA200a in peripheral CD4+ of HT patients might cause an increase of proinflammatory Th1 cytokines which damage thyroid cells causing them to increase their production of miRNA200a. The decrease of miRNA200a in CD8+ T-cells might contribute to the pathological recognition of thyroid specific cellular antigens (i.e., TPO and Tg) ultimately resulting in thyroid destruction by CD8+ cells [3]. In GD, the decrease of miRNA200a in CD4+ T-cells in GD patients might also cause an increase of proinflammatory Th1 and may on this way contribute to the disease.

The other miRNA candidates tested did not show significant differences between different AITDs, even though evidence exists for their involvement in immune regulation.

In the 3 study groups, no significant group differences with regard to patients' mean age were found. In the HT group women were overrepresented. This likely reflects the 8 to 10-fold increased susceptibility of women to this disease [38]. So far, however, there is no data supporting an influence of gender to the expression of miRNAs. Further on all GD patients were under therapy with methimazole or carbimazole. It might be possible that thyrostatics may affect the expression of miRNA in GD patients. Further studies will be needed to compare GD patients under treatment to untreated GD patients.

## Conclusion



GD and HT are common human disorders whose exact aetiology is still unknown. Some miRNAs are suspected to influence autoimmune diseases. This study could confirm significant variations of miRNA146a1, miRNA200a1 and miRNA155 in PBMCs, CD4+ and CD8+ T-cells of patients suffering from GD and HT in vivo. These data may help to better understand the cause of the autoimmune processes leading to AITDs as we proved significant variations of miRNAs responsible for posttranscriptional gene regulations in the causative cells. More efforts are required to understand the relevance of these miRNA variations in AITDs and to clearly identify the target genes.

**Disclosure Statement:** No funding was received for this work from any organization. The authors declare that no competing interests exist.

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