Autoimmune Thyroid Diseases

Traces of Viral Infection

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A cause is something that makes a difference.

Mervyn Susser, American Journal of Epidemiology, 1991.

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Abbreviations

AITD	Autoimmune thyroid disease
CAR	Coxsackie-and adenovirus receptor
EBV	Epstein-Barr virus
FFPE	Formalin-fixed, paraffin-embedded
FT4	Free thyroxine
GD	Graves' disease
GWAS	Genome wide association studies
HHV-6	Human herpesvirus 6
HLA	Human leukocyte antigen
HT	Hashimoto's thyroiditis
IFN	Interferon
IHC	Immunohisotchemistry
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK cell	Natural killer cell
PAMP	Pathogen-associated molecular patterns
PKR	Protein Kinase R
STAT1	Signal trandsucer and activator of transcription 1
T1D	Type 1 diabetes
TPO-Ab	Thyroid peroxidase antibody
TRAb	Thyrotropin receptor antibody
TSH	Thyroid stimulating hormone

In the thesis text, both MHC and HLA will be used. HLA is used when the particular study is based on human cells, and MHC when referring to animal cells.

Summary

Autoimmune thyroid diseases, which mainly consist of Graves' disease and Hashimoto's thyroiditis, are common and affect quality of life considerably. Yet, no cures exist and insights into the underlying disease mechanisms are limited. However, new evidence points to viral infections as possible triggers for autoimmune thyroid disease.

The aims for this doctorate thesis were to explore the viral trigger hypothesis by analyzing immunoproteins involved in the antiviral defense system and to confirm the presence of common viruses in thyroid tissue from patients with Graves' disease and Hashimoto's thyroiditis.

Standard immunohistochemistry and immunofluorescence protocols were applied on formalin-fixed, paraffin-embedded thyroid tissue samples from 118 patients and controls in order to detect HLA class I, signal transducer and activator of transcription 1 and protein kinase R, which are all important antiviral proteins and enzymes. Additionally, the enteroviral capsid protein VP1 was assessed. Common viruses, including parvovirus B19, human herpesvirus 6, enterovirus, Epstein-Barr virus and cytomegalovirus, were analyzed with a novel technique based on pre-enrichment in cell culture, before viral DNA and RNA detection with PCR.

We found significantly more HLA class I in thyroid tissue derived from AITD patients than in controls. In addition, HLA class I and signal transducer and activator of transcription 1 were found within the same thyroid cells. Likewise, VP1 was colocalized with protein kinase R, indicating an active antiviral immune response. Enterovirus, as well as several highly common viruses such as human herpesvirus 6 and parvovirus B19, are frequent in thyroid tissue from both patients and controls.

HLA class I upregulation is a defining feature of autoimmune thyroid disease which supports the viral trigger hypothesis. Moreover, several common viruses are able to infect the thyroid gland. However, further studies, with additional techniques are needed to verify our findings. Our results may be transferable to other autoimmune diseases, and in its broadest impact might be a step on the way to prevent autoimmune disease.

Sammendrag

Autoimmune stoffskiftesykdommer, som hovedsakelig består av Graves sykdom og Hashimotos tyreoiditt, rammer mange og gir redusert livskvalitet. Vi vet ikke hva som forårsaker sykdommene, og det finnes ingen kurativ behandling. Derimot dukker det stadig opp nye bevis på at virusinfeksjon i skjoldbruskkjertelen kan utløse autoimmun tyreoideasykdom.

Jeg har i mitt doktorgradsarbeid utforsket virushypotesen ved å undersøke forekomsten av virus, samt ekspresjon av antivirale immunproteiner i tyreoideavev fra pasienter med Hashimotos tyreoiditt og Graves sykdom.

Vi undersøkte tyreoideabiopsier fra en kohort bestående av 118 pasienter med autoimmun tyreoideasykdom og kontroller. Ved bruk av immunohistokjemi på formalin-fikserte, parafin-innstøpte vevsprøver, så vi på HLA klasse I, signal transducer and activator of transcription 1 og protein kinase R, som alle er viktige antivirale immunproteiner. I tillegg så vi på enteroviruskapsidprotein VP1. Vi tok i bruk en helt ny metode for å påvise virus i hurtigfryst tyreoideavev, hvor virus blir dyrket i beriket cellekultur før påvisning av viralt DNA og RNA med PCR.

HLA klasse I var signifikant oppregulert i vev fra pasienter med Graves sykdom og Hashimotos tyreoiditt sammenlignet med kontrollene. Vi påviste HLA klasse I i de samme cellene som signal transducer and activator of transcription 1. Protein kinase R var uttrykt i de samme cellene som VP1, noe som indikerer en aktiv, antiviral immunrespons. Både enterovirus og flere andre utbredte virus, som human herpesvirus 6 og parvovirus B19, ble funnet i tyreoideavev fra både pasienter og kontroller.

Oppregulering av HLA klasse I kjennetegner autoimmune tyreoideasykdommer, og støtter virushypotesen. I tillegg har vi vist at flere hyppig forekommende virus infiserer tyreoidea. Men flere studier med andre metoder trengs for å verifisere disse funnene. Vi tror at resultatene fra studiene er overførbare til andre autoimmune sykdommer, og i sin videste forstand et steg på veien mot forebygging av autoimmunitet.

List of papers

1. Weider T, Richardson SJ, Morgan NG, Paulsen TH, Dahl-Jorgensen K, Hammerstad SS. **Upregulation of HLA Class I and Antiviral Tissue Responses in Hashimoto's Thyroiditis.** Thyroid. 2020;30(3):432-42.

2. Weider T, Richardson SJ, Morgan NG, Paulsen TH, Dahl-Jorgensen K, Hammerstad SS. **HLA Class I Upregulation and Antiviral Immune Responses in Graves Disease.** J Clin Endocrinol Metab. 2021;106(4):e1763-e74.

3. Weider T, Genoni A, Broccolo F, Paulsen TH, Dahl-Jorgensen K, Toniolo A, Hammerstad SS. **High Prevalence of Common Viruses in Thyroid Tissue**. Submitted.

The papers are referred to by their Arabic numeral throughout the thesis. Paper 3 has been published, with minor revisions, in Frontiers in Endocrinology after the thesis was approved.

Figures and Tables

1 Introduction

1.1 Background

The thyroid gland is a small, highly vascularized endocrine organ located on the front of the neck. Its main function is to produce the thyroid hormones thyroxine (T4) and triiodothyronine (T3), which regulates metabolic processes at the cellular level. Thyroid diseases are typically suspected when there is a clinically apparent under- or overproduction of thyroid hormones. Thyroid hormone dysfunction is highly common, with a reported prevalence of 12% in some populations, and is most frequently caused by autoimmune thyroid disease (AITD), toxic adenomas or iatrogenic complications (1). A disruption in the thyroid hormone function can be detrimental and, though rarely, mortal. Other pathological processes such as nodular growth or cancerous tumors may affect the thyroid gland without causing a disturbance in the thyroid hormone function. In iodine replete countries, over- and underproduction of thyroid hormones, i.e. *hypo*thyroidism or *hyper*thyroidism, are most often caused by AITD. Hashimoto's thyroiditis (HT) and Graves' disease (GD) are the two main AITDs, but postpartum thyroiditis and silent thyroiditis are also generally included in the AITD spectrum.

The prevalence of AITD varies, especially since the definition of AITD differs, but a prevalence of 2 to 10% has been reported (1, 2). While normally not life-threatening, the AITDs cause morbidity and reduced quality of life (3, 4). The AITDs are characterized by a break of tolerance to thyroid specific proteins, which results in the generation of thyroid autoantibodies and lymphocyte infiltration of the thyroid gland.

The autoimmune thyroid diseases have been known for centuries, but no cures exist and the etiology remains unknown. However, some AITD risk genes have been identified (5, 6). Still, twin studies reveal that AITD variance is not explained solely by genetic susceptibility; environmental factors play a part as well. Smoking, excess iodine intake, infections and stress are associated with AITD (7). While several epidemiologic studies report an increasing incidence of thyroid dysfunction, it remains to be verified if this is a result of more thyroid hormone testing (8, 9).

Emerging evidence point to viral infections as potential triggers for AITD. This doctoral thesis includes papers in which we aim to support this theory. The thesis consists of three separate papers investigating the presence of antiviral immune response proteins and virus in thyroid tissue. Understanding the etiology of AITD is the first step towards a cure, or even better; prevention.

Figure 1. Edvard Munch, *The Seducer* (1913). Photo © The Munch Museum. One can only speculate if the object of the artist's affection has a goiter.

1.2 Hashimoto's Thyroiditis

The distinctive features of HT was first described by the Japanese surgeon Hakaru Hashimoto in 1912 (10), but the paper did not receive recognition until two decades later. Hashimoto´s thyroiditis became the first autoimmune disease recognized when Roitt and colleagues described thyroid antibodies in 1956 (11, 12).

Hashimoto's thyroiditis is the most frequent autoimmune endocrine disease, affecting 2 to 6% of the population, with women being five to ten times more likely to be affected than men (13). Fatigue, weight gain, cognitive impairment and general malaise are the most common symptoms of hypothyroidism. Hypothyroidism in its severe form, also known as myxedema coma, can cause fatal hypotension, hypothermia, hypoglycemia and severely altered mental status.

Lymphocytic infiltration is one of several pathologic changes found within the thyroid gland in HT. A thyroid antigen specific CD4+ T cell (helper T cell) is activated by triggering factors currently unknown. Once activated, an immune response that results in T and B lymphocyte migration to the thyroid gland, induction of antibody-secreting B cells (plasma cells) and thyroid cell destruction occur. In its late stage this results in insufficient production of thyroid hormones, and clinically evident hypothyroidism, which requires permanent replacement therapy with synthetic thyroxine (levothyroxine). Despite adequate thyroxine replacement therapy, as assessed by serum thyroid hormone levels, many patients still experience symptoms (14, 15).

The hallmark of HT is the generation of autoantibodies to thyroglobulin and/or thyroid peroxidase (TPO-Ab); a thyroid specific protein and enzyme, respectively. Thyroglobulin is a glycoprotein produced by the follicular cells of the thyroid (thyrocytes), and serves as the major precursor for the synthesis of thyroid hormones. Thyroid peroxidase oxidizes iodide to iodine, which is added onto tyrosine residues on thyroglobulin, which makes the backbone of the thyroid hormones. Thyroid dysfunction in the presence of serum TPO-Ab or thyroglobulin antibodies is diagnostic for HT. Overt, untreated hypothyroidism is rarely seen in Norway today, most likely due to accessible laboratory services (16).

Approximately 80% of patients with AITD have thyroid antibodies (17). Whether or not these antibodies have any pathologic effect per se is currently unknown (17). Moreover, thyroid antibodies in euthyroid individuals are highly common, with some studies reporting a TPO-Ab prevalence of 14% in women and 3% in men (13, 18). The TPO-Ab prevalence increases with age, reaching 26% in middle-aged women according to one study (19). Nonetheless, the presence of thyroid antibodies increases the risk of developing

AITD. The odds ratio for developing overt hypothyroidism is eight in TPO-Ab positive women and 25 in TPO-Ab-positive men (20, 21).

1.3 Graves' Disease

Graves' disease affects 20 to 50 persons per 100 000 per year (22, 23), with a prevalence of approximately 1% (24). Women are four to six times more likely to be affected than men (23, 25). Palpitations, tachycardia, weight loss, tremor, reduced physical capacity, anxiety and emotional liability are amongst the most common symptoms of hyperthyroidism. Approximately 50% of patients with GD experience some form of eye involvement however severe eye disease is more rare (5-10%) (26). Thyroid dermopathy, especially on the shins (pretibial myxedema), and digital clubbing are more unusual extra-thyroidal manifestations. Severe forms of hyperthyroidism, also known as thyroid storms, can cause arrhythmias, fever and shock. Although rare, thyroid storms or severe thyrotoxicosis can be fatal.

The current first-line GD treatment is antithyroid drugs (methimazole, carbimazole and propylthiouracil), which inhibit the thyroid hormone production. Most patients are treated for one to two years inducing remission of the disease, but approximately 50% experience relapses and require several rounds of treatment. Frequent relapses, eye disease or aggressive disease are some of the arguments for opting for permanent treatment, such as thyroidectomy or radioiodine ablation.

The TSH receptor antibody (TRAb) is an immunoglobulin G1 autoantibody of oligoclonal origin, produced by plasma cells mainly within the thyroid, and is pathognomonic for GD. Normally, the TSH receptor is stimulated by the pituitary hormone TSH, which regulates the rate of thyroid hormone production. In GD, however, TRAb stimulates and activates the TSH receptor without pituitary interference (reviewed in (27)). Activating TRAb mimics TSH through similar, but not identical signaling pathways, and the result is thyroid hormone excess, i.e. hyperthyroidism (28). However, blocking and neutral TRAbs exist as well, leading to hypothyroidism or no thyroid dysfunction, respectively (29-31). Other thyroid antibodies, such as thyroglobulin antibodies and TPO-Ab are also frequently found in GD, but are believed to be a result of epitope spreading due to thyroid cell damage. No current specific pathologic roles of these antibodies are known (32).

Normally, the immune system is constantly presented with self-antigens, but does not elicit an immune response; a feature called immunological tolerance. In GD, a thus far unestablished initiating event generates a breach in immunological tolerance. The CD4+ T

cells reactive to components of the TSH receptor somehow escape thymic and peripheral tolerance induction, and an immune attack against the TSH receptor ensues. These TSH receptor - reactive CD4+ T cells activate an immune cascade via cytokines and interleukins, resulting in T cell differentiation and B cell activation. Activated B cells differentiate into TRAb-producing plasma cells. The results of this immune cascade are inflammation and lymphocyte infiltration of the thyroid gland. In addition to hyperplasia and hypertrophy, the thyrocytes are believed to be directly involved in the immune response as well. Thyrocytes release chemokines, which attract immune cells (33). Furthermore, both human leukocyte antigen (HLA) class II and the B cell activating receptor CD40 have been found in thyrocytes (34-36).

The initiating incident that prompts the immune response is poorly understood, but environmental factors and genetic susceptibility alike contribute to AITD etiology.

1.4 Genetic Susceptibility to Autoimmune Thyroid Disease

Bartels did the first scientific report on familial clustering of thyroid diseases in 1941, and recorded a familial incidence of 47% in patients with hyperthyroidism (37, 38). In spite of being two clinically distinct diseases, HT and GD are observed within the same families (39, 40), and shared susceptibility genes have been found (41). Moreover, there are reports of cross-phenomena and conversion between the two diseases (42, 43). However, both genome-wide association studies (GWAS) and twin studies point to less genetic overlap between GD and HT than previously thought (44, 45).

The concordance rate between monozygotic twins ranges from 21% to 35% for GD and from 29% to 55% for HT (45-48). In comparison, the dizygotic twin concordance rates (3-2% for GD and 0-1%for HT) differ significantly from the monozygotic concordance rates, which indicate a strong genetic component in GD and HT (45-48). With the use of structural equation modelling, the concordance rate has been used to calculate heritability, yielding a heritability of 63 to 79% for GD and 65% for HT (45, 46). This suggests that 65% of the HT variation in the specific population studied is explained by genetic factors. A heritability above 50% indicates a strong genetic component (49).

Most AITD susceptibility genes are involved in immune system regulation and T cell activation or de-activation. Some of these immunoregulatory genes confer risk for several autoimmune diseases, while thyroid specific genes, such as TSH receptor and thyroglobulin are unique for AITD.

Genes within the HLA locus are thus far the ones with the strongest AITD association, and was the first AITD susceptibility locus identified in 1974 (50). Later, this finding was specified as the class HLA class II alleles HLA-B8 and HLA-DR3 (51, 52). The DR3 frequency is 40-55% in GD patients and 15-30% in the general population, yielding a relative risk of 3 to 4 for people with HLA-DR3 (52). The HLA-DR allele that confers the highest risk for both GD and HT is HLA-DRβ1-Arg74; an HLA-DR with an arginine at position 74 in the β-chain (53, 54). One speculates that this specific pocket amino acid sequence conveys an AITD risk due to high affinity for thyroid peptides involved in the thyroid autoimmunity pathogenesis, such as thyroglobulin and the TSH receptor (55-58). Furthermore, HLA class I genes have also been associated with AITD (59-62).

Protein tyrosine phosphatase nonreceptor type 22 (PTPN22), cytotoxic T lymphocyte associated protein 4 (CTLA-4) and TSH receptor are other AITD susceptibility genes discovered with candidate gene studies (56, 63, 64) . Later, tag- single nucleotide polymorphism case-control studies, GWAS and immunochip data have revealed more AITD susceptibility genes. So far, six to nine common variants (defined as allele frequency >1%) with great effect (loosely defined as a two-fold disease risk compared to the background population) have been confirmed by GWAS across various populations (Table 1) (59-62, 65). Some susceptibility loci are unique for certain populations, and new and rare variants are discovered regularly (66-68).

Despite several large GWAS (>500 000 people included) being performed, the risk variants identified so far only confer relatively small increases in disease risk and account for an estimated 10-20% of the heritability (69, 70). This phenomenon is seen in many complex diseases, and is often referred to as the "missing heritability" problem. Various explanations for this observation exist. One explanation is that there are probably still many unidentified variants with small effect that requires very large populations to be discovered. Moreover, the risk loci or genes do not account for gene-gene interactions and gene-environment interactions. Additionally, a substantial proportion of risk variants lie in non-coding DNA (71). Finally, epigenetic influence may contribute as well and hypermethylation of several GD susceptibility genes have been reported (72).

Table 1. Major AITD susceptibility genes

Gene	Locus	Protein function	Associated diseases	Reference
TSHR	14q31.1	Central tolerance	GD	59-61,65
PTPN22	1p13	T cell activation	GD,HT T1D SLE RA	62,65
FCRL3	1q23.1	T cell activation	GD, HT SLE RA MS	59-61,65
CTLA-4	2q33.2	T cell activation	GD, HT T1D SLE RA MS PBC IBD Celiac disease Addison's disease Sjogren's disease Systemic sclerosis Myasthenia gravis	59,61,62,65
RNASET2	6q27	Cytokine modulation	GD T1D RA IBD Vitiligo	59,61,65
HLA class I and II	6p21	Antigen presentation	GD, HT T1D SLE RA Celiac disease Bechet's disease Myasthenia gravis	59-62

TSHR, thyrotropin receptor; PTPN22, protein tyrosine phosphatase nonreceptro type-22; FCRL3, Fc receptor-like 3; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; RNASET 2, ribonuclease T2; SLE, systemic lupus erythematosus; RA, rheumathoid artritis; PBC, primary biliary cirrhosis; IBD, inflammatory bowel disease. Adapted and modified with permission from (73).

1.5 The Environment and Autoimmune Thyroid Disease

Iodine status was one of the first environmental factors recognized to contribute to AITD incidence. A change from iodine deficiency to iodine sufficiency increases the GD and HT incidence (74, 75), and GD is slightly more common in iodine replete areas (76). In mild to moderately iodine deficient populations, iodine fortification of salt increases the incidence of overt hypothyroidism; whether or not caused by autoimmunity, however, is unknown (77).

Smoking is the environmental factor most strongly associated with GD. In addition, severe and protracted disease and eye involvement are more frequent in smokers (78, 79). The pathomechanism behind this association is not fully explained. The association between radiation and thyroid cancer is well-established; however, radiation of the thyroid may increase AITD risk as well. An increased incidence of thyroid antibodies was reported after the Nagasaki bombing and the Chernobyl nuclear fall-out (80). The mechanism might be the same as the one seen in radioablation of the thyroid; cellular damage lead to a release of thyroid antigens, which increases the risk of thyroid autoantibodies (81-83).

Like most other autoimmune diseases, AITD is more common in women than in men (13). Theories involving both sex hormone differences and X chromosome inactivation exist (reviewed in (84)). Many autoimmune diseases debut, get better (or worse) during periods characterized by dramatic hormonal changes, such as puberty, pregnancy and menopause; which strengthen the sex hormone theory. An observation that supports the X chromosome theory, on the other hand, is that men with Klinefelter syndrome (XXY karyotype) have the same risk for systemic lupus erythematosus and Sjogren's syndrome as women (85). Moreover, women with true monosomy X (40-50% of women with Turner syndrome) have a significantly higher AITD risk than other women, suggesting that a gene on the long arm of the X chromosome may play a role in AITD pathogenesis (86).

Some studies show that parity and pregnancy may influence AITD risk. A longer reproductive span increases the risk for HT (87). In GD, disease suppression is frequently seen in pregnancy, while increased incidence and exacerbation of existing disease is seen postpartum (88-91). Maternal immune tolerance to the fetus is necessary to maintain a pregnancy, and is thought to explain the amelioration seen in some autoimmune diseases during pregnancy. One suggested mechanism for the high AITD risk postpartum is sensitizing of maternal antigens by intrathyroidal fetal cells (microchimerism). Indeed, thyroid microchimerism is more frequent in women with AITD than in women with other thyroid diseases (92, 93). Still, the AITD prevalence is not higher in multiparous women than in nulliparous women (94-98).

Stressful life events are also associated with GD (99). In fact, one of the first acknowledged reports of GD by Parry in 1825, described the disease in a young woman who had been thrown out of her wheelchair at high speed, and thus suffered trauma and shock (100).

The gut microbiota and its role in modulating immune response has seen intense investigation the last years, and variations in the microbiome have been associated with autoimmune diseases, such as type 1 diabetes (T1D), rheumatoid arthritis, multiple sclerosis (MS) and Crohn's disease (101-104). Moreover, a number of studies have revealed that alterations in the gut microbiome can be found in patients with both HT and GD (105-111).

Several immune-modulating therapies developed for cancer and severe, debilitating autoimmune diseases, such as MS, are associated with a high incidence of AITD. Especially alemtuzumab, a humanized, monoclonal antibody directed against lymphocyte and monocyte cell surface antigen CD25, carries a considerable AITD risk. Alemtuzumab was developed as a leukemia therapy, but has been used for relapsing remitting MS the last decade (112, 113). Its action is lymphocyte depletion with following immune reconstitution. The reported thyroid autoimmunity incidence is 30-50%, with GD accounting for 70-80% of all AITD incidents (114-117). Other immune reconstitution therapies, such as highly active antiretroviral therapy is also associated with a high frequency of AITD (118). Additionally, immune checkpoint inhibitors used in cancer treatment, such as ipilimumab (CTLA-4 inhibitor) and nivolumab (programmed cell death protein 1 inhibitor) are associated with AITD (119, 120). Finally, non-specific immune stimulatory therapies, such as interleukin 2 and IFNα, used in metastatic renal cancer and hepatitis C, respectively, also carry a high thyroid autoimmunity risk (121, 122).

A final environmental factor that seems to influence the AITD incidence is seasonal variation. GD debut and GD relapse are more frequent during spring and summer (123, 124). Since many viral infections fluctuate during the year, this supports the viral trigger hypothesis.

1.6 Virus and the Immune System

The antiviral defense is anchored in the innate and adaptive immune system. The innate immune system is non-specific and has no memory. Cell receptors of the innate immune system recognize traits called pathogen-associated molecular patterns (PAMPs), which are shared by several classes of microbes. Double stranded RNA (dsRNA), which is found in a number of viruses, and lipopolysaccharide - a bacterial cell wall component, are examples of PAMPs. The PAMPs are usually components of highly preserved molecular structures vital for survival and replication. Engaged innate cell receptors activate transcription factors that promote genes encoding cytokines and enzymes involved in the antimicrobial functions of phagocytes and other immune cells. Furthermore, the cytokines and enzymes serve as second signals, which are required for the T and B cells of the adaptive immune system in order to elicit a strong immune response. See (125) for further details on the innate immune system.

The lymphocytes of the immune system can be classified into three main categories; B lymphocytes, T lymphocytes and natural killer (NK) cells. T lymphocytes mainly fall into two subtypes; the CD4+ T cell and the CD8+ T cell (cytotoxic T cell). Whilst the CD8+ T cell mainly executes its function in the innate immune system, the CD4+ T cell serves as a mediator between the innate and the adaptive immune system. The CD4+ T cell activates the B cells of the adaptive immune system, and stimulates the production of antigen-specific antibodies by the B cells (126). Both CD8+ T cells and NK cells contain granules that kill infected target cells. However, the NK cell is not specific, but is set off by an imbalance between activating and inhibitory receptors (127, 128)

Interferons (IFNs) are important antiviral signaling cytokines. Type 1 IFNs, which include IFNα and IFNβ, are secreted by several cell types when infected by viruses, but the main source is the plasmacytoid dendritic cell. Type 1 IFNs bind to IFN receptor on infected cells or adjacent uninfected cells, and activate a signaling pathway that inhibit viral replication and destroy the viral genome. Moreover, type 1 IFNs enhance the NK cells' capacity to eradicate infected cells. Type 2 IFN consists of IFNγ only. In contrast to the type 1 IFNs, which are produced by a variety of cells, type 2 IFN is only produced by immune cells, such as T cells and NK cells. Type 2 IFN kills virus directly, and stimulate the upregulation of major histocompatibility complex (MHC) class I and II. See (129) for a comprehensive review on interferons.

The HLA complex

The MHC complex (HLA complex in humans) is a cluster of genes on chromosome 6 which encodes membrane glycoproteins involved in antigen presentation and processing, as well as other immune system proteins. Due to a large number of alleles at the various loci, with many of them in strong linkage disequilibrium, the HLA genes are the most polymorphic in the human genome (130). The HLA complex contains approximately 260 genes and nearly 40% of them are assumed to have an immune function (131). The HLA class I molecule complex consists of a transmembrane α-chain and a β2-microglobulin chain, and is present in all nucleated cells.

HLA class I regulates the cytotoxic effects of NK cells and CD8+ T cells, and acts as an essential surveillance mechanism against invading pathogens. HLA class I presents intracellular protein fragments and viral antigens on the cell surface to the extracellular environment. The CD8+ T cells recognize the HLA class I-viral antigen complex, and subsequently destruct the infected cell by releasing IFNs as well as igniting a cascade of other immune defense mechanisms. The HLA class I molecule presents self-proteins as an inhibitory signal to NK cells; i.e. the expression of HLA class I is crucial in order to prevent NK-mediated destruction. HLA class II presents peptides from the extracellular environment, such as bacterial components, to immune cells.

HLA class I presentation of viral products is a crucial step in the host's antiviral defense. As a result, countless strategies to manipulate this process are deployed by several viruses. Cytomegalovirus, for example, can downregulate HLA class I in order to prevent CD8+ T cell attacks (132). On the other hand, a number of viruses induce upregulation of HLA class I; a feature believed to be a way of escaping NK cells (133-141).

Hanafusa and Bottazzo found an aberrant expression of HLA class II in AITD thyroid tissue, and argued that thyroid cells are complicit in inducing the autoimmune response (36). The same research group established that thyrocytes can reverse their polarity and serve as antigen-presenting cells via the HLA class II pathway, thus demonstrating that antigens can be sensed by circulating T cells (142, 143). Moreover, viral infection has been shown to upregulate MHC class II in rodent thyroid cells (144). HLA class I, on the other hand has been less investigated, both in autoimmunity in general, and in thyroid autoimmunity in particular.

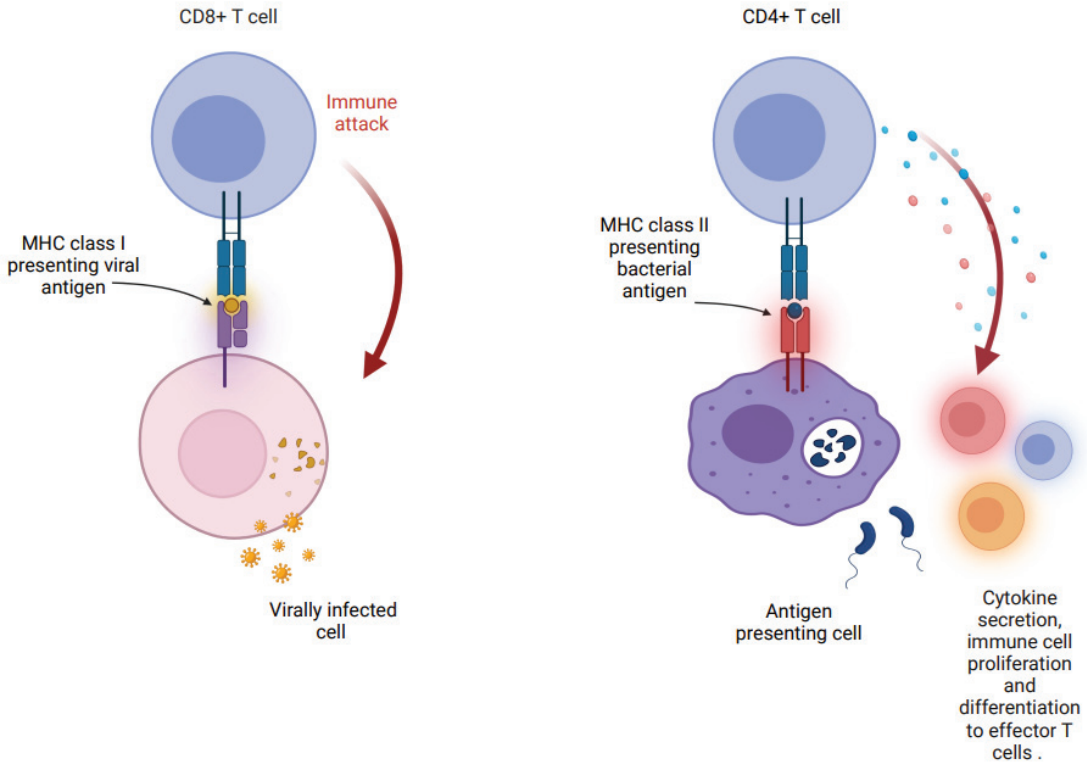

Figure 2. Schematic presentation of MHC class I and MCH class II. While MHC class I is expressed on all nucleated cells, MHC class II is expressed by professional antigen-presenting cells only; i.e. dendritic cells, macrophages and B cells. MHC class I normally presents endogenously derived antigen (from virus and self), while MHC class II presents exogenously derived antigen (bacterial fragments). MHC, major histocompatibility complex. Created with BioRender.com.

STAT1 and PKR

Signal transducer and activator of transcription 1 (STAT1) is an antiviral protein activated by IFNs (145, 146). Virus initiates STAT1 via IFNγ. When IFNγ stimulates the IFNγ1 and IFNγ2 receptors, STAT1 tyrosine is phosphorylated, which in turn leads to translocation of STAT1 into the nucleus where it starts DNA transcription of IFN-inducible genes (147-150). Protein kinase R (PKR) expression is prompted by IFNs, and activated by dsRNA, which is a product of viral genome replication (151, 152). PKR is an enzyme (serine/threonine kinase), which after activation, first autophosphorylates then phosphorylates the alpha subunit of eukaryotic initiation factor 2α. When phosphorylated, eukaryotic initiation factor 2α inhibits protein translation, thus inhibits the virus' ability to produce its protein. Indeed, one of the IFN-stimulated genes transcribed in response to activated STAT1 is PKR (153). Moreover, there is a feedback mechanism not entirely understood, but PKR modifies and interacts with STAT1 (Figure 5.). Nevertheless, studies have shown that PKR-deficient cells display reduced and defective STAT1-dependent functions, including transactivation and IFN-stimulated phosphorylation of STAT1 (154).

1.7 Autoimmunity and the Viral Infection Theory

The onset of disease following infection was properly acknowledged in the aftermath of the second world war with the description of rheumatic fever and Guillain-Barré syndrome (155, 156). Later, associations between viral infections and various autoimmune diseases were made, with links between virus and T1D and MS standing out as the strongest (157-160).

The main theories on how viral infection can cause autoimmunity are as follows: Infections prompt the innate immune system to release cytokines and induce expression of costimulators on antigen-presenting cells which are necessary for T cell activation (bystander activation). Moreover, microbial peptides can have structural homology with self-antigens (molecular mimicry). The classic example of molecular mimicry is a *Streptococcus pyogenes* antigen that resembles myosin, a cardiac muscle protein, which leads to immune attack on myosin and subsequent rheumatic heart disease (161, 162). Proteins derived from infectious agents, such as *Borrelia burgdorferi* show structural homology with the TSH receptor, thus making the molecular mimicry theory a plausible mechanism for GD (163). Finally, local damage induced by infection can reveal previously sequestered proteins, releasing potentially new autoantigens (epitope spreading). Infection leads to the production of inflammatory cytokines and expression of costimulators on antigen-presenting cells, thus attracting immune cells to the infected area and decreasing the threshold for self-attack. Further details on proposed mechanisms in infection and autoimmunity are revised in (164).

Subacute thyroiditis was described in the late 1800s and the association with viral infection was suggested a half century later (165, 166). Infections with mumps, measles, coxsackievirus, adenovirus and Epstein-Barr virus (EBV) have all been associated with subacute thyroiditis (167). Now it is generally acknowledged that subacute thyroiditis is a transient inflammation of the thyroid due to viral infection, and not considered an autoimmune disease. Even though most people recover entirely from subacute thyroiditis, subsequent bouts of GD, persistent thyroid autoimmunity and a rise in thyroid autoantibodies occur (168-171).

When it comes to thyroid autoimmunity, there have been many attempts at unveiling a triggering infectious agent, and the bacteria Yersinia enterocolitis was one of the first suggested as an etiological agent in GD in the 1970s (172, 173). A number of viruses have been associated with AITD. Human herpesvirus 6 (HHV-6) has been detected in thyroid tissue from patients with both HT and GD (174, 175). Parvovirus B19 has also been associated with AITD (176-180). Even the highly common EBV has been linked to GD (181,

182). Moreover, children with AITD more often have EBV antibodies than the general population (183). While subacute thyroiditis and non-thyroid illness seem to be frequently occurring in severe acute respiratory syndrome coronavirus 2 infection (184-186), reports on thyroid autoimmunity are also emerging (187-190). Finally, thyroid cells have been successfully infected in vitro by several viruses, including lymphocytic choriomeningitis virus, HHV-6 and hepatitis C virus (191-193).

One of the viruses with the strongest association with thyroid autoimmunity is enterovirus. Enteroviruses are diverse and cause a variety of diseases. The *Enterovirus* genus is separated into 12 species (*Enterovirus A-H, Enterovirus J* and *Rhinovirus A-C*). Coxsackievirus, echovirus and poliovirus are examples of well-known enteroviruses. Transmission is either respiratory or fecal-oral, and the primary site of replication is the gastrointestinal tract. But enterovirus can spread and infect a range of tissues and organs. Most enteroviral infections cause unnoticeable disease. Viral aseptic meningitis, neonatal sepsis-like disease, encephalitis, pericarditis and myocarditis, however, are important exceptions (194, 195). In addition, big outbreaks of respiratory disease and hand-foot-mouth disease occur from time to time (196, 197). Moreover, evidence of persistent enterovirus infection of the pancreas, which ultimately leads to autoimmune destruction of beta cells, is emerging (198-200). Other autoimmune diseases, such as celiac disease is also linked with enterovirus (201). Furthermore, enterovirus has been associated with thyroid disease; both subacute thyroiditis and AITD (202-205).

Viral tropism and pathogenesis are largely determined by access to cell surface receptors. The diverse disease spectrum caused by enteroviruses might be partly explained by the many and varied receptors with which they engage. Nineteen enterovirus receptors have been identified thus far (206), one of which is the coxsackie- and adenovirus receptor (CAR). Coxsackie- and adenovirus receptor is a transmembrane glycoprotein with two immunoglobulin-like extracellular domains, and is the main entry route for adenovirus and coxsackievirus B (belonging to the *Enterovirus B* species) (207). Coxsackie- and adenovirus receptor is expressed in many tissues, with a particular high expression in in the pancreas, heart, prostate, testis and the small intestines (208) . In addition to allowing viral entry, CAR regulates more complex functions involving endothelial tight junctions and epithelial permeability (209, 210). Four of five known isoforms of CAR bind enterovirus, but only two of the isoforms (CAR-SIV and CAR-TVV) contain a transmembrane domain, suggesting an ability to mediate an intracellular infection (211). RNA sequencing recently showed that the SIV-isoform was the most expressed CAR isoform in the thyroid (212).

2 Aims

2.1 General Aims

The autoimmune thyroid diseases, comprising HT and GD, are the most common endocrine autoimmune disorders. Yet, no cures exist and the etiology remains largely unknown. While a strong genetic component has been established, environmental factors are believed to play a part as well. Recent studies, including results from the thyroid tissue sample collection used in this thesis, point to virus as potential triggers for AITD. The general aims of this study were to elucidate AITD etiology and to explore the virus trigger hypothesis in particular. A better understanding of the activating events in autoimmune disease can generate cures or even better; prevention.

2.2 Specific Aims

In order to explore the viral trigger hypothesis, HLA class I expression is interesting due to its crucial function in the antiviral immune response. Genes involving antigen presentation including, HLA class I genes, confer autoimmunity risk. Moreover, several viruses are known to manipulate the HLA class I expression in order to establish themselves within the host. Research on HLA class I expression in autoimmune diseases is limited, and especially so for AITD.

Viral infections prompt a complex immune cascade involving the innate and adaptive immune system. Signal transducer and activator of transcription 1, which promotes transcription of several antiviral genes, and PKR which inhibits viral protein translation are two key players in the antiviral defense system.

Many viruses give rise to minor infections in infancy, and have a high seroprevalence in the adult population. While serological studies of these viruses are abundant, the research on highly prevalent viruses and their ability to infect the thyroid gland is scarce.

The specific aims of this study were to explore the presence of antiviral immunoproteins and confirm previous findings of enterovirus in thyroid tissue from a previously collected cohort consisting of patients with GD and HT. We applied immunohistochemistry and immunofluorescence in formalin-fixed, paraffin-embedded thyroid samples to assess

expression of enteroviral capsid protein VP1 (VP1), CAR and HLA class I. We analyzed colocalized expression of PKR with VP1 and STAT1 with HLA class I.

Additionally, enterovirus and HHV-6 were assessed with a novel method based on pre-enrichment of thyroid tissue in cell cultures, while the highly frequent viruses EBV, parvovirus B19, CMV and common gastroenteric viruses were assessed by PCR.

3 Methods

3.1 Study Population

Ninety-four AITD patients (48 GD and 46 HT) and 24 controls were previously recruited at the endocrinological outpatient clinic at Aker, Oslo University Hospital (204, 213). Patients referred to the clinic due to thyroid autoimmunity were invited to join the study. Twenty-six GD patients suffered from chronic disease (median disease duration of 24 months) and 22 GD patients were newly diagnosed (within the last three months). Thyroid tissue samples were collected by core needle biopsy in patients with newly diagnosed disease, and from surgical specimens during thyroidectomy in patients with chronic disease. All but three chronic GD patients received antithyroid drugs upon study inclusion (22 on carbimazole and one on propylthiouracil) whilst none of the recent-onset GDs had started with antithyroid drugs. Moreover, ten chronic GD patients received glucocorticoid therapy before study inclusion. Hashimoto's thyroiditis was defined as TPO-Ab>34 kIU/L with or without thyroid dysfunction. The HT group was divided into three subgroups according to thyroid hormone levels: overt hypothyroidism (TSH ≥ 10 mIU/L and/or active treatment with thyroxine) (n=15), subclinical, non-treated hypothyroidism (3.6 mIU/l ≤ TSH ≤10 mIU/l and FT4 8-20 pmol/l) (n=14) and non-treated prethyroiditis (TSH ≤3.6 mIU/l and FT4 8-20 pmol/l) (n=17). Thyroid tissue samples from 24 patients undergoing neck surgery for other reasons than AITD, i.e. thyroid tumors or parathyroid adenomas, served as controls. Serum TRAb, TPO-Ab and thyroglobulin antibodies were measured in all control subjects in order to rule out pre-existing or unrecognized thyroid autoimmunity. The whole cohort was used in papers 1 and 2. In paper 3, however, the whole cohort could not be included, due to limited availability of frozen tissue samples. All patient data were anonymized and encrypted according to present data protection rules.

Table 2. Characteristics of Patients and Controls at Study Inclusion

	Control group (n=24)	Graves' disease (n=48)	*P value**	Hashimoto's thyroiditis (n=46)	*P value***
Duration *(months)*	-	12.0 (0.0-24.0)	-	0.0 (0.0-6.0)	-
Age *(years)*	52.0±14.4	44.0±13.1	*0.021*	43.4±12.8	*0.014*
Female *n(%)*	22 (91.7)	42 (87.5)	*0.596*	42 (91.3)	0.955
TSH *(mIU/l)*	1.4 (0.7-2.5)	0.0 (0.0-0.0)	*<0.001*	4.0 (1.6-7.0)	*<0.001*
FT4 *(pmol/l)*	13.1 (12.1-14.5)	24.2 (16.7-45.8)	*<0.001*	13.5 (10.5-14.2)	*0.565*

Duration is time from diagnosis to biopsies. *P controls versus Graves' patients; **P controls versus Hashimoto's patients.
Values presented as median and interquartile ranges, mean±standard deviation, numbers and percentages.
Reference ranges: TSH 0.5-3.6 mIU/l, FT4 8-20 pmol/l. FT4, free T4.

3.2 Tissue Biopsies and Sample Preparation

Thyroid sampling from HT patients and newly diagnosed GD patients were obtained in local anesthesia, using a Magnum® reusable biopsy device and a 16 G needle (Bard Medical, Bard peripheral Vascular, Inc.) All biopsies were taken from the right lobe and performed by a single surgeon with ultrasound guidance. Two patients experienced minor bleeding after the procedure, but no major adverse events were recorded. In patients with chronic GD, the thyroid tissue samples derive from surgical specimens taken at the time of total thyroidectomy. In addition to thyroid tissue sampling, all patients underwent blood sampling at the same day as tissue sampling. Samples from the 24 control subjects derive from tissue sampling during neck surgery for other reasons than thyroid autoimmunity. The specimens were split in two parts, were one part was fixed in 10% neutral-buffered formalin and embedded in paraffin within 24 hours. The other part was snap-frozen in liquid nitrogen, and stored at -80°C. The formalin-fixed, paraffin-embedded (FFPE) tissue samples were routinely cut into 3 mcm slides, mounted on slides and stored at 4 °C.

3.3 Immunohistochemistry and Immunofluorescence

Formalin-fixed, paraffin-embedded thyroid tissue slides from 94 AITD patients and controls were immunostained for VP1 and HLA class I using a standard IHC protocol. Antigens were unmasked by heating in 10mM citrate buffer (pH 6.0), in a pressure cooker in a microwave oven (800W) for 20 minutes, followed by 20 minutes of cooling at room temperature. Sections were blocked with 10% goat serum and primary antibodies diluted

in Dako REAL antibody diluent (Agilent S202230). The Dako anti-enteroviral VP1 (5D8/1 clone) was used at a dilution of 1:1400 (55ng/ml) for 30 minutes. The HLA class I primary antibody (Abcam class I HLA [EMR8/5] Ab70328) was used at a dilution of 1:1500 for one hour. Primary antibodies were visualized using the Dako REAL EnVision Detection system (Agilent K5007).

Due to limited resources, and a shortage of STAT1 antibody (Abcam stopped production), only a subgroup of samples was immunostained sequentially with STAT1, followed by HLA class I. The same subset of cases was stained simultaneously for PKR and VP1. Primary antibodies were detected using species-specific secondary antibodies conjugated to either AlexaFluor® 488 or AlexaFluor® 555 as appropriate (Invitrogen, UK).Full antibody details and conditions are listed in Supplementary (S) Table 1. We analyzed CAR-SIV (Abcam Ab 100811 antibody) on ten thyroid tissue samples acquired from a collection available at the University of Exeter, UK. No information on any prior AITD was available for these samples, but judging by microscopy alone, the samples were deemed normal, i.e. most likely no pre-existing thyroid disease.

3.4 Viral Detection in Pre-Enriched Cell Cultures

In paper 3, a novel method for viral detection developed by our collaborators in Varese, Italy was employed in the search for enterovirus and HHV-6 (214). Five cell lines were tested for the main enterovirus- and HHV-6 entry factors using indirect IF (S.Table 2), proving them permissive for these specific viruses. Fifty-four snap-frozen thyroid tissue biopsies from the same thyroid sample collection used in papers 1 and 2 were made into homogenates, and cultured with the five cell lines. We extracted RNA (enterovirus) and DNA (HHV-6) after two to three serial passages. RNA was converted to DNA using a commercially available RNA transcriptase, before running end-point PCR with enterovirus- and HHV-6 specific primers (S.Table 3). We confirmed positive results with Sanger sequencing. Amplicons of viral isolates were identified based on best matches in public databases. In addition to PCR, we detected virus using IF in cell monolayers from the same pre-enriched cell cultures. Three antibodies directed at the VP1 capsid protein (9D5, 6E9/2 and 5D8/1) and two antibodies directed at the viral 3D polymerase (3D-02 and 3D-05) were applied for enterovirus detection. For HHV-6, an antibody directed at the HHV-6 specific 140 kDA capsid polypeptide was applied.

For the nine other viruses searched for in paper 3, we used real-time PCR. Due to the limited size of the snap-frozen thyroid tissue samples, we used a whole genome

amplification method in order to obtain sufficient amounts of DNA templates allowing for numerous PCR tests. A commercially available whole genome amplification method was run according to the manufacturer's protocol (Sigma-Aldrich, Milano Italy). RNA was converted to DNA as described above. The primers used to run real-time PCR for CMV, EBV, parvovirus B19, HCV, adenoviruses F40-F41, astrovirus species 1-8, norovirus genogroups I and II, rotaviruses and sapoviruses are listed in S. Table 4.

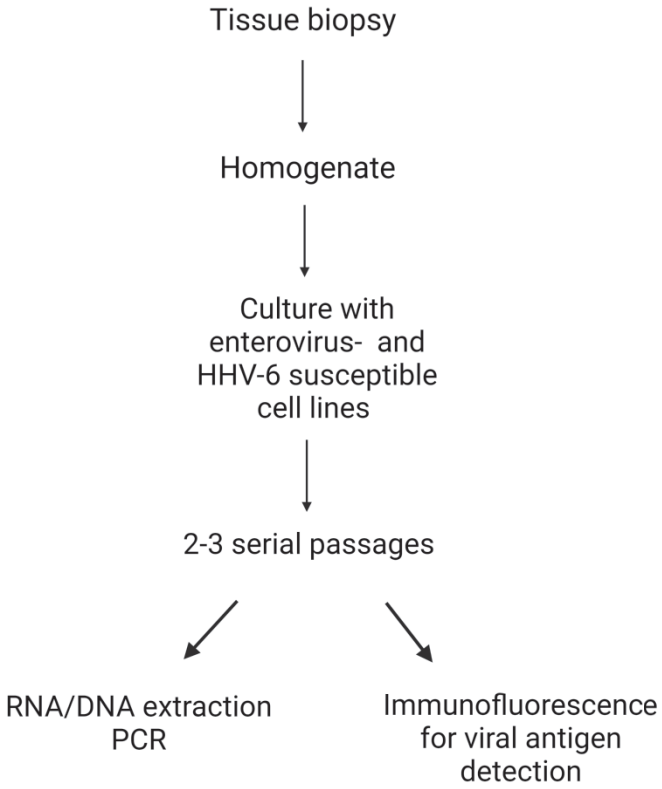

Figure 3. Model of the integrated procedure used for deriving virus strains from thyroid tissue. Thyroid biopsy tissue homogenates were cultured with five cell lines with proven enterovirus- and human herpesvirus 6 (HHV-6) entry factors. After 2-3 serial passages, RNA (enterovirus) and DNA (HHV-6) were extracted, before viral genome detection by PCR. For each case, the expression of enterovirus and HHV-6 antigens in cultured cells were evaluated by immunofluorescence with a panel of enterovirus and HHV-6 antibodies. Created with BioRender.com.

3.5 Statistical Analyses

Data analyses were performed using IBM SPSS Statistics version 25 (IBM SPSS Inc., Armonk, NY, USA: IBM Corp.) and GraphPad Prism 7.02 (GraphPad Software, Inc., La Jolla, CA, USA). We considered p values less than 0.05 significant. Continuous data are presented as means and standard deviations if normally distributed and with medians and interquartile ranges if the distribution is skewed. Categorical data are presented as numbers (proportion).

All three papers have populations of a small sample size, thus some variables are highly affected by outliers. Because of that, both mean and median have been used to present the central tendency in papers 1 and 2. The distribution was evaluated with the Shapiro-Wilk's test for normalcy, skewness and kurtosis and by graphical methods (Q-Q plots and histograms).

Statistical significance of differences between groups was calculated with the independent samples T-test if fulfilling criteria of normality, and with the Mann-Whitney U test if not. In all three papers, several of the outcomes were dichotomous, and we used Pearson's chi square test to identify whether the proportion of positive results in the AITD group and the control group were significantly different.

Associations between clinical patient features and results were explored using binary logistic regression if one variable (dependent) was dichotomous (i.e. VP1-positivity or HLA class I - positivity), Pearson's correlation for continuous data with linear relationship and Spearman's correlation for ordinal data, with monotone relationships.

3.6 Ethics and Funding

The study was approved by the Norwegian South-Eastern Regional Committee for Medical and Health Research Ethics (REK no. 1.2006.1950) and performed according to the Helsinki Declaration. The nature and implications of the study were explained to all patients in an accessible manner orally, as well as per written information sheets. Potential adverse events were outlined, and the risk of bleeding after thyroid biopsy was particularly emphasized. For patients undergoing neck surgery, there was no additional health risk in being included in the study, since they were already scheduled for operation. Details on data storage and potential future use were also provided. Before committing to the study, patients were encouraged to read through the information at home. All included patients provided written informed consent.

This PhD thesis was supported by the following sources: The South-Eastern Norwegian Regional Health Authority (project no. 39887). The Faculty of Medicine at the University of Oslo provided a travelling grant for the research stay in Exeter, UK.

4 Main results –summary of papers

4.1 Paper 1

Upregulation of HLA Class I and Antiviral Tissue Responses in Hashimoto's Thyroiditis

This paper reports HLA class I upregulation in thyroid tissue from HT patients compared to controls; the presence of enterovirus in the same groups; and the interaction between viral immune response proteins STAT1 and PKR with HLA class I and VP1, respectively. The paper also reports the findings of CAR in thyroid cells.

Significantly more HT samples were HLA class I positive (31 out of 46 [67.4%]) compared to controls (5 out of 24 [20.8%]) ($p<0.001$). Moreover, the Allred score, which is a semi-quantitative score taking both intensity and proportion of immunostaining into account, was significantly higher in the HT samples (3.9±3.1) than in the controls (0.5±0.9) ($p<0.001$). We found more VP1 in the HT samples (20.1±16.4%) than in controls (14.9±10.5%), albeit not significantly so. Coxsackie- and adenovirus receptor is expressed in a granular way in thyroid cells. A select number of tissue samples were stained sequentially with immunofluorescence for the immune response protein and enzyme, STAT1 and PKR, respectively. STAT1 was colocalized with HLA class I and PKR was colocalized with VP1. Finally, we confirmed the presence of CAR in thyroid cells.

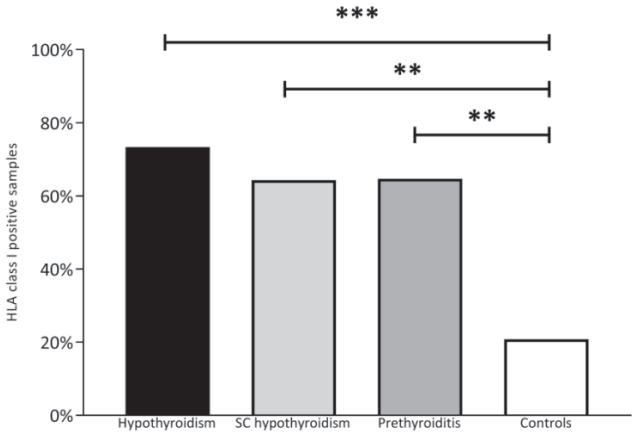

Figure 4. Proportion of samples with HLA class I positivity within the three clinical subgroups of HT. **p≤0.01, ***p≤0.001. SC, subclinical.

4.2 Paper 2

HLA Class I Upregulation and Antiviral Immune Responses in Graves' Disease

This paper reports HLA class I overexpression in thyroid tissue from GD patients compared to controls; the presence of enterovirus in the same groups; and the colocalization of antiviral immune proteins STAT1 with HLA class I and PKR with VP1. A significantly larger proportion of thyroid tissue samples in the GD group was HLA class I positive (25 out of 49 (52.1%)) compared to the control group (five out of 24 (20.8%)) (p=0.011). The HLA class I expression score, taking both intensity and proportion of immunostaining into account, was significantly higher in GD patients (3.1±3.3) than in controls (0.5±0.9) (p<0.001). We found VP1 in more GD samples (29 of 48 [60.4%]) than in controls (10 of 24 [41.7%]). This difference was only significant when comparing chronic GD (19 of 26 [73.1%]) to controls (10 of 24 [41.7%]) (p=0.025). A significantly higher number of $VP1^+$ thyrocytes were found in the VP1-positive GD samples (median 40.0% [19.0%-80.9%]) than in the VP1-positive control samples (median 11.7% [7.7%-18.1%]) (p<0.001). A fraction of all the thyroid tissue samples was stained sequentially with immunofluorescence, for the antiviral immune response protein and enzyme, STAT1 and PKR, respectively. STAT1 and HLA class I were found within the same thyroid cells and PKR and VP1 were also colocalized within thyroid cells.

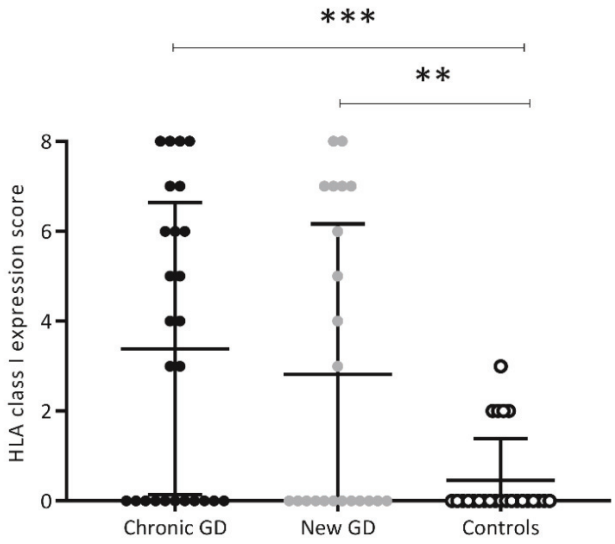

Figure 5. HLA class I expression score in GD, taking both immunostaining intensity and proportion of stained thyrocytes into account (maximum score 8, and lowest score 0). Bars represent mean and standard deviation. **p≤0.01, ***p≤0.001.

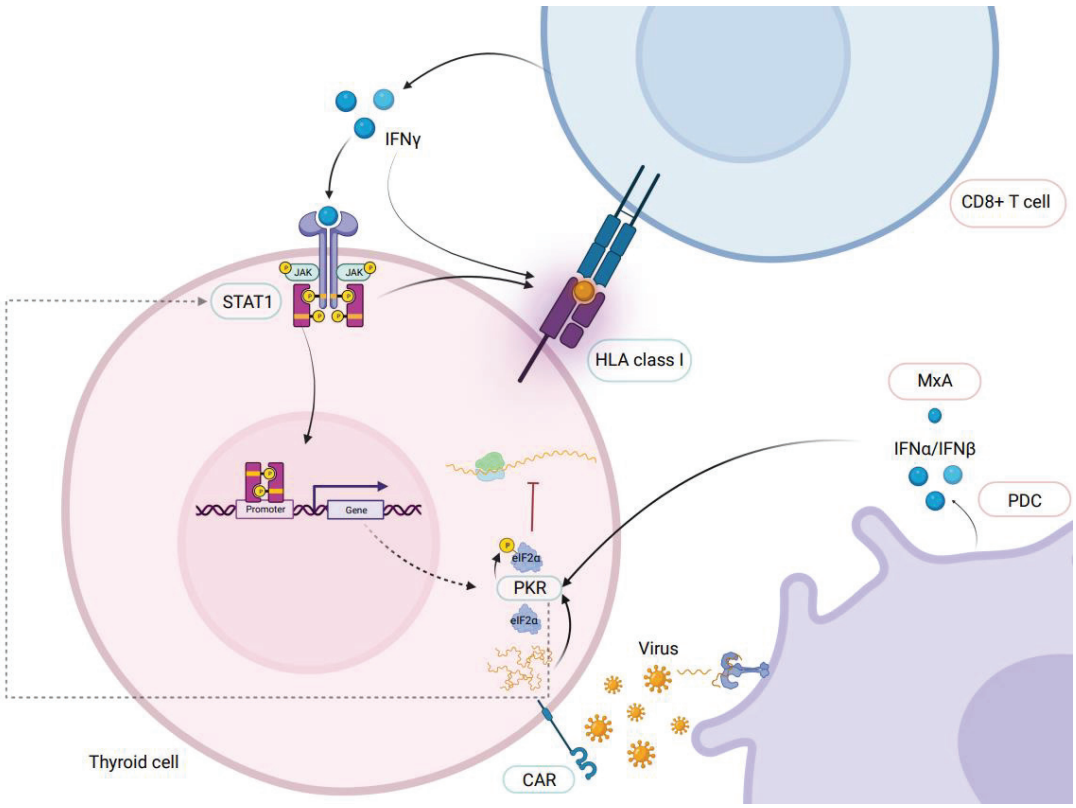

Figure 6. A schematic model of findings from papers 1 and 2 (green ring) in this thesis, and previous papers (red ring) from the same thyroid tissue cohort. Virus enters the thyroid cell via CAR. The viral product dsRNA activates PKR, which phosphorylates eukaryotic initiation factor 2α, which inhibits viral translation. PKR interacts with the STAT1 pathway. Viral nucleic acids activate PDCs, which produce IFNα and IFNβ, which induce PKR expression. Viral fragments are presented to the CD8+ T cell by HLA class I, and this leads to IFNγ production. IFNγ activates STAT1, and increases HLA class I expression. Activated STAT1 relocates to the nucleus and induce antiviral genes, one of which is PKR. PDCs, MxAs and CD8+T cells have been found in the same thyroid tissue samples in previous papers (213, 215). CAR, coxsackie- and adenovirus receptor; dsRNA, double-stranded RNA; PKR, protein kinase R; STAT1, signal transducer and activator of transcription 1; PDC, plasmacytoid dendritic cell; IFN, interferon; HLA, human leukocyte antigen; MXA, myxovirus resistance protein A. Created with BioRender.com.

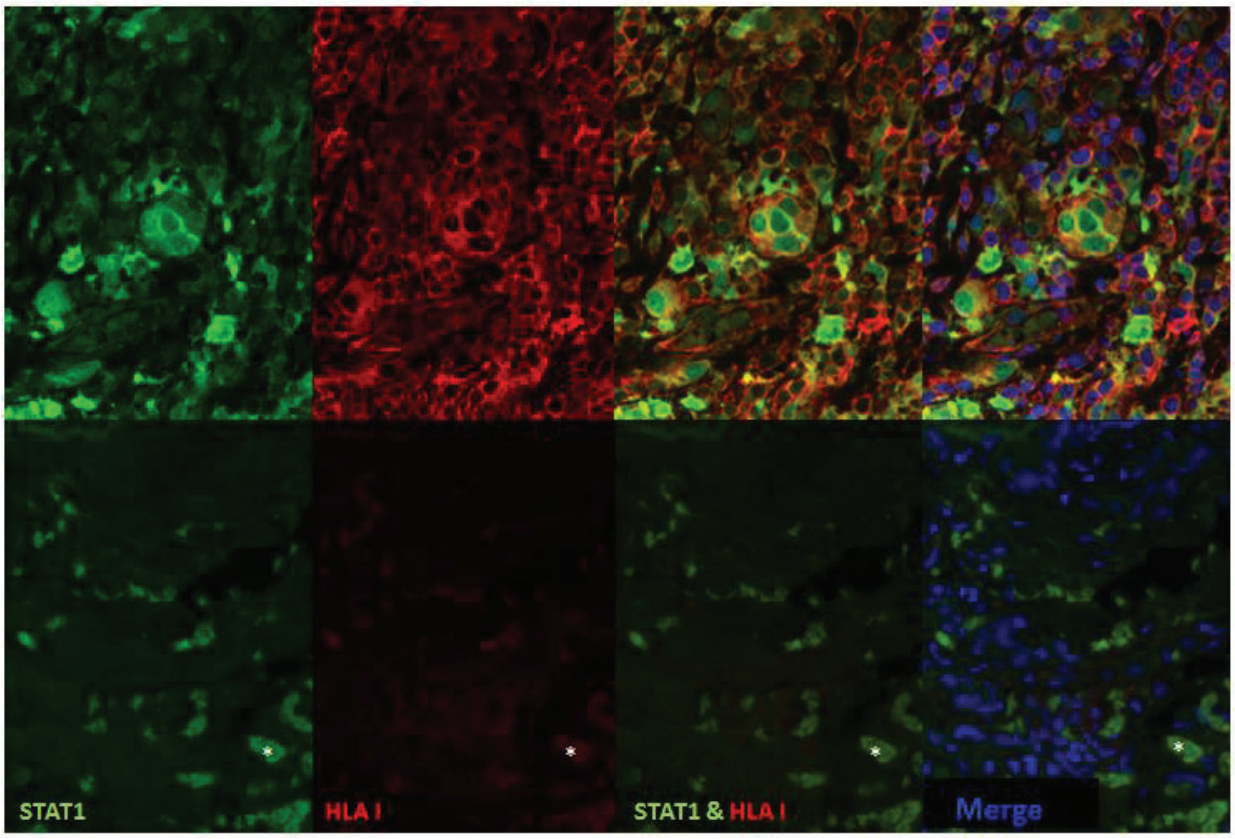

Figure 7. Nuclear and cytosolic STAT1 was found in thyroid cells and was colocalized with HLA class I. The upper panel shows an example of STAT1 (green) and HLA class I (red) immunofluorescence staining in GD thyroid tissue. Control sample with negative STAT1 and HLA class I staining is seen in the lower panel. Merged images with blue nuclear DAPI staining. All scale bars at 25 μm. STAT1, signal transducer and activator of transcription 1; PKR, protein kinase R; DAPI, 4′,6-diamidino-2-phenylindole.

4.3 Paper 3

High Prevalence of Common Viruses in Thyroid Tissue

This paper reports the results from analyses of several commonly occurring viruses within thyroid tissue samples from both AITD patients and controls. This is the first study to assess the presence of multiple viruses at once in a large thyroid tissue cohort.

In this study, snap-frozen thyroid tissue samples from 53 AITD patients and controls carefully selected from the previously collected cohort used in paper 1 and 2. Tissue samples of a sufficient size were chosen and analyzed with a novel method using pre-enrichment of thyroid tissue homogenates before PCR for HHV-6 and enterovirus. Parvovirus B19, EBV, cytomegalovirus, hepatitis C virus and five common gastroenteric viruses were detected with whole-genome amplification and real-time PCR.

Enterovirus (49.1%), HHV-6 (32.1%) and parvovirus B19 (22.6%) were found in a large proportion of the samples. Moreover, very few samples had no evidence of virus. There were no statistically significant differences in detection rates for each virus when comparing AITD patients to controls. For enterovirus, there was a slight discrepancy in detection rate between PCR and IF, with IF being more sensitive. For HHV-6, on the other hand, the detection rate was concordant.

Table 3. Viral detection rate in paper 3.

	No virus	At least one virus	Three viruses	Enterovirus	HHV-6	Parvovirus B19	EBV
All n=53	13 (24.5%)	40 (75.5%)	4 (7.5%)	26 (49.1%)	17 (32.1%)	12 (22.6%)	3 (5.7%)
GD n=20	5 (25.0%)	15 (75.0%)	2 (10.0%)	10 (50.0%)	6 (30.0%)	4 (20.0%)	2 (10.0%)
HT n=15	4 (26.7%)	11 (73.3%)	2 (13.3%)	9 (60.0%)	4 (26.7%)	3 (20.0%)	1 (6.7%)
CTRL n=18	4 (22.2%)	14 (77.8%)	0 (0.0%)	7 (38.9%)	4 (38.9%)	5 (27.8%)	0 (0.0%)

GD, Graves' disease; HT, Hashimoto's thyroiditis; CTRL, controls; HHV-6, human herpesvirus 6; EBV, Epstein-Barr virus.

5 Discussion

The quality of a study is assessed by its validity, the theory of which has been attributed to Campbell and Stanley (216). The internal validity is defined as to which degree the findings represent the truth in the study population, and is affected by design, methodological errors (such as selection and sample bias), analysis and interpretation. The following section describes factors in the study that affect the validity of our findings.

5.1 Methodological Considerations

Patient population

Selection bias is a culprit in medical research, and an inherent possible flaw in cross-sectional studies. Most studies have some degree of selection bias, as the individuals who participate might be different from the ones that decline.

In addition to the inherent flaw of selection bias, our study has additional bias due to the selection of HT patients and controls. Patients and controls were recruited from an endocrinology outpatient clinic at Oslo University Hospital. Normally, HT patients are not treated by endocrinologists; rather they are treated by their primary care physicians. Thus, HT patients referred to specialist care might have more severe symptoms than the general HT population. GD patients, on the other hand, are normally seen by an endocrinologist shortly after onset of the disease, thus this patient group is probably representative for all GD patients.

The controls were not drafted from a general population, i.e. not randomly selected. Ideally, control groups should be similar to the general population. In our study, the controls were chosen out of convenience since they were undergoing neck surgery for thyroid tumors and primary hyperparathyroidism, thus study enrollment did not pose any additional risk or discomfort. Antibodies were measured to ensure no undiagnosed AITD existed and the tissue sample was taken from healthy tissue, with a wide margin away from the pathological lesion for which the patient underwent surgery. Nevertheless, one cannot exclude the possibility that the control subjects differ from the general population. Moreover, there might be differences in viral exposure in the control group compared to the general population. In fact, some studies suggest that there might be an association between viral infections and thyroid cancers (217, 218). Seven out of 24 (29.2%) controls underwent thyroid surgery due to tumors, while the remaining part of the control

group underwent surgery due to parathyroid adenomas. We do not know, however, how many of the tumor patients ended up with a cancer diagnosis post-surgery. No significant differences within the two control groups in HLA class I expression, VP1 detection or viral detection rate were found. The control group was smaller than both the HT group and the GD group.

Formalin-fixed, paraffin-embedded samples

Formalin-fixed, paraffin embedded samples are used to preserve tissues for extended periods of time while conserving the morphology and cellular details of the tissue. Moreover, FFPE samples are a cost- and time efficient way of preparing and storing tissues. For these reasons hematoxylin and eosin stained FFPE samples are still the most commonly used tissue preparations by pathologists. Formalin-fixed, paraffin embedded sample preparation protocols are not globally standardized, which is a drawback when comparing methods. It is important to note that immunoreactivity in FFPE samples decrease over time, but optimized antigen retrieval protocols can partially compensate for this (219). Virus detection in FFPE tissue samples is technically challenging due to RNA degradation, DNA fragmentation and cross-linking. Immunohistochemistry and in situ hybridization are the only widely used methods in FFPE samples. However, low-copy viral DNA can be detected in FFPE samples (220). Moreover, studies have shown that it is possible to amplify virus RNA from FFPE samples (221, 222). The influenza A virus from the great pandemic in 1918-1919 was genetically characterized from RNA isolated from a 1918 FFPE lung tissue sample (223). This is promising in regards to archived, historical material. Nevertheless, virus isolation in cell culture is still considered the gold standard for virus detection. But the procedure is laborious, time-consuming and cannot detect latent, non-replicating virus, which is why virus detection relies on more readily available methods such as nuclear acid amplification methods.

Sample selection

In papers 1 and 2, samples from the entire cohort were used. For each individual, several FFPE samples were available. Tissue quality affects immunostaining results, thus we carefully chose samples of good quality, as judged by size and tissue integrity. Therefore, some degree of selection bias cannot be excluded. Moreover, tissues used in the FFPE samples were acquired in two different ways; by surgery or by core needle biopsy. Chronic GD and control samples were attained surgically, whilst samples from patients with HT and newly diagnosed GD were acquired by core needle biopsy. Tissue samples from surgeries were generally larger in size, which implies an increased chance of immunopositive staining. Since all controls were gained by the surgical method, this could skew our results with more positively stained cells in the controls than in the patient

samples. However, this bias was addressed by taking the number of thyrocytes in the sample into account; positively stained thyrocytes were counted alongside the total number of thyrocytes in ten consecutive counting grids (0.058 mm2) on 400X magnification, thus yielding a fraction of positive thyrocytes. Interestingly, in paper 1, where all HT samples were acquired by core needle biopsy and controls by surgery, there were more immunopositive HLA class I and VP1 cells in the HT samples than in the control samples. Due to both limited availability of the STAT1 antibody and limited time at the study facility (University of Exeter), only a select number of samples were stained sequentially for HLA class I and STAT1 and PKR and VP1. We chose samples from cases with sufficient FFPE slides of good quality (one for HLA class I and STAT1 and one for VP1 and PKR).

Immunohistochemistry

Immunohistochemistry has been, and still is, extensively used in both clinical and experimental settings since its invention in the 1940s (224). The method, which is considered qualitative and descriptive, applies antibodies to find specific antigens. Other antibody-based techniques are western blot, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay and flow cytometry. Unlike abovementioned techniques, IHC has the unique advantage of identifying both the specific antigen and its geographical position within the tissue.

The immunostaining was done by one scientist (TW), thus avoiding the issue of inter-assessor variability, but introducing the possibility of systematic errors. In fact, there are several issues with the IHC method that are prone to information bias, i.e. an error in the measurements of the exposure, outcome or its confounders (225). First of all, pre-analytical factors can have an effect on IHC results. More specifically, both tissue preparation (fixation and paraffin embedding) as well as the epitope retrieval method for the given protocol, may affect the final outcome. Moreover, the IHC protocol can yield false positive signals, which can be due to pigment, non-specific background signal, endogenous peroxidase, inappropriately high antibody solution, drying artifact and antibody cross-reactivity with other proteins (226). Few, if any antibodies show absolute specificity in all conditions. On the other hand, false negative results can occur due to unfitting epitope retrieval, a non-optimized protocol or an antibody solution which is too dilute. Finally, the sensitivity and specificity of each antibody is often given by the producer. Thus, usually each laboratory needs to establish their own protocols based on whatever tissue they are testing and under which conditions. For more clinically relevant antibodies, studies with non-commercial actors that give accessory information on each antibody´s performance in given settings usually exist.

The lack of quality assurance is a shortcoming of the IHC method. Unlike quantitative assays used clinically for analyte measurements in for example serum, there are neither quality assurance nor universal reference standards for the IHC method. Still, the sensitivity and specificity of the primary antibody in an IHC protocol can be measured in a liquid based assay, such as ELISA, but is not possible in tissue sections. Detection systems facilitate the visibility of the antibody-antigen reaction. These systems use amplification in order to detect antigen, thus it is a non-linear system, and the amount of antigen is not possible to establish. In the future, however, the overall qualitative method of IHC might be developed into a quantitative tissue-based immunoassay, called in-situ proteomics (227).

Enteroviral capsid VP1 is one out of four recognized enteroviral capsid proteins, and makes viral attachment to the target cells possible (228). Enteroviral capsid VP1 has a conserved immunodominant region, which is shared by multiple enterovirus serotypes. In papers 1 and 2, the monoclonal antibody 5D8/1 was applied. This antibody is directed against the peptide sequence EIPALTAVE in the VP1 protein, and has been used to reveal pancreatic enterovirus infection in T1D patients (158, 229). There has been some debate about the 5D8/1-specificity in pancreas, but several studies have proved its robustness and replicability (230-232). In thyroid tissue on the other hand, the antibody has not been extensively tested. However, FFPE samples from the same thyroid tissue collection were previously stained with the 5D8/1 antibody at a different lab with a slightly different protocol, but yielded overall comparable results (204), demonstrating reproducible and robust results.

Immunohistochemical analyses

The immunostaining and interpretation were performed in a blinded fashion. The interpretation of an IHC staining is done by humans, and is in nature a qualitative analysis. The staining was done by one person (TW), which limits the variability in how the protocol was performed. The analyses of the staining were done by two assessors (TW and co-author on papers 1 and 2 SJR). An overall agreement on scoring was reached, and in the cases with discrepancies, disagreements were solved by consensus. Even though the analyses were done blindly, pathological tissue changes in GD and HT makes observer bias impossible to be completely ruled-out.

The slides were analyzed by light microscopy on 400X magnification. The proportion of VP1 and HLA class I were assessed by counting all positively stained thyrocytes along all thyrocytes in ten consecutive counting grids (0.058 mm2) on 400x magnification, which gave us a percentage of positively stained thyrocytes. For the VP1 assessment, we did not take intensity into account.

For HLA class I, on the other hand, both intensity and proportion were evaluated, using the semi-quantitative Allred score. The Allred score was developed in 1999, in order to assess estrogen receptor status in breast carcinomas, and is still used by clinical pathologists today (233, 234). We chose to apply this scoring system due to an observed a difference in staining intensity. As already explained in methodological considerations though, the staining intensity may not reflect how "much" HLA class I there is, as IHC protocols are non-linear. Since the IHC method is not suitable for quantifying the amount of a given protein, the titles of papers 1 and 2, are not strictly speaking correct, because the difference in HLA class I expression between AITD patients and controls is not readily quantifiable. Nevertheless, qualitatively there was a significant difference between patients and controls and more cells in the patient samples were positive than in the control samples. Moreover, we observed a relationship between intensity and proportion. Finally, the control samples were of a higher quality, since they derived from surgical samples. Taking this into account, the chances of finding HLA class I were actually higher in the control samples. Thus the information bias that might occur due to sample selection could be skewed in favor of false positive controls.

Table 4. Allred score used in HLA class I immunoreactivity scoring

Score	Percentage of cells stained		**Score**	Immunostaining intensity
0	No	HLA class I^+ cells	**0**	None
1	<1%	HLA class I^+ cells	**1**	Weak
2	1-10%	HLA class I^+ cells	**2**	Intermediate
3	11-33%	HLA class I^+ cells	**3**	Strong
4	34-66%	HLA class I^+ cells		
5	>67%	HLA class I^+ cells		

The total score is the proportion score + the intensity score, yielding a score from 0 to 8.

Detection of enterovirus and HHV-6 in thyroid tissue following enrichment in cell culture

Persistent enterovirus infection of cultured cells exhibits unique features, one of which is low virus titers and a lack of cytopathic effect in cell cultures (235). In these specific circumstances, the amount of viral RNA accessible for PCR primers is too small and reduces the method's sensitivity. Previous studies have shown persistent enterovirus infection of pancreatic beta cells. The viral load is however minor and few cells in total are infected (158). Moreover, as with other RNA viruses, enterovirus have a high mutation rate, which means that reference enterovirus strains obtained decades ago might differ considerably from present enterovirus strains. Additionally, new enterovirus species are

frequently found and sometimes exhibit divergence in the 5´untranslated region, a highly conserved region, thus used as a target region for many detection assays (236-238). When it comes to enterovirus detection, serologic methods are not widely used due to the lack of shared antigens between enterovirus.

In paper 3, we applied a new method developed by our collaborators in Varese, for detection of viruses in tissues and cells with a low viral load. Cell lines were exposed to thyroid tissue homogenates, in a hope that virus in thyroid tissue could be transferred to the permissive cells and replicate. The method is based on pre-culture of enterovirus and HHV-6 susceptible cell lines with the tissues or cells in which we want to detect the virus. This method yields significantly enhanced virus detection compared to direct assays of biological samples (214). The method is new, and needs verification over time.

PCR is a universally validated method, allowing rapid and sensitive pathogen detection. Its use is accessible, affordable, sensitive and specific. However, you need pre-defined targets (primers) and the PCR product (amplicon) relies on primer specificity. We used five enterovirus primer pairs and one HHV-6 primer pair. Due to the high sensitivity, the risk for contamination in the use of all PCR methods is high. The most significant risk for contamination in the PCR process comes from PCR carryover products; that is previously detected amplicons can be implemented in a new analysis and amplified. Furthermore, there are risks for contamination between samples and between nucleic acids. In this new, integrated approach we used end-point PCR, which requires post-PCR product handling; a process which is a potential source for carryover contamination. In the detection of viruses other than enterovirus and HHV-6, we used qPCR, which eliminates the post-handling source of contamination.

Cell cultures can be contaminated by microbial pathogens in the lab or, albeit rare, when acquired, i. e. at the repository. However, the cell lines used in paper 3 were all acquired from recognized repositories (The American Type Culture Collection and The European Collection of Animal Cell Cultures), which undergo strict quality controls. The most frequent microbial contamination is by mycoplasma, and was assessed with a commercial detection kit. Other bacterial infections are easily detectable due to destruction of the cell cultures. Some virally infected cell cultures do not produce any cytopathic effect. Although rare, viral contamination occur from time to time, and is in some cases only detectable with virus-specific PCR assays (239). The enterovirus RNA genomes detected after cell-enrichment, showed at least six different enterovirus strains, making contamination unlikely. For the HHV-6 detection, we used one primer pair only, targeting a highly conserved genomic region common to both HHV-6A and HHV-6B. The amplicons produced in the HHV-6 PCR did not allow discrimination between different HHV-6 strains.

Finally, we detected virus in in only three out of 19 leukocyte controls (EBV, enterovirus and HHV-6). Leukocytes from these controls were cultured with the cell lines, and subsequently processed and handled identically to the cells cultured with the thyroid tissue homogenates. We also ran the direct PCR for the other viruses on these controls. The low viral detection rate in the control samples makes contamination unlikely.

5.2 Discussion of Results

In our papers, we show evidence of viral infections in thyroid tissue, both indirectly via upregulated HLA class I and immunoproteins, and directly with findings of VP1 in papers 1 and 2, and viral DNA or RNA from several virus in paper 3. In the following section I will discuss the results of papers 1 and 2 together, and paper 3 separately.

HLA class I

HLA class I is crucial in viral immune defense, and genes involving antigen presentation confer susceptibility to autoimmunity. Due to both its key function in host recognition of virus and the antigen presentation function of HLA class I, its expression in tissues with autoimmune disease is of high interest.

HLA class I upregulation was a prominent feature in both the HT patient group and the GD patient group. The proportions of HLA class I positive samples and the HLA class I score were significantly higher in all AITD subgroups compared to controls.

Enhanced HLA class I expression in AITD has previously been shown, although our study is, as far as we know, the most extensive. Hanafusa and Bottazzo were credited for the concept of faulty antigen presentation as a key event in the autoimmune processes. Their seminal work from 1983 showed both aberrant HLA class I and class II expression in GD (36). This paper, which included thyroid specimens from 26 GD patients, one HT patient, nine non-toxic nodular goiter patients and 11 controls, showed increased HLA class I staining in the majority (62%) of GD patients and in the one HT patient. No other studies, except ours, have looked at such a vast collection of thyroid specimens. Like us, they used an immunostaining principle to detect HLA class I as well, albeit in three different sample preparations (frozen cryostat sections, follicle suspensions and monolayers). The findings of Hanafusa and Bottazzo were in fact reproduced by Bagnasco, who also found aberrant HLA class I and II expression in thyroid tissue from HT and GD patients (240). However, this study only included two controls. Likewise, Huang and colleagues verified that HLA class I was upregulated in thyrocytes from 22 HT patients (241). In contrast to the two

other groups that shared these findings, Huang confirmed the results with PCR in addition to immunoassay-based methods (western blot and IHC). Additionally, MHC class I is an important pathomechanistic factor in the rat model of AITD (242, 243). More recently, a group in Shanghai explored the observation of better prognosis in HT patients with papillary thyroid carcinoma. They found that papillary thyroid carcinoma patients with concomitant HT had a higher HLA class I expression than those without HT (244-246). They believe that the increased HLA class I expression seen in HT enhances immunosurveillance and anti-tumor properties.

Virus can modulate HLA class I, and both up- and downregulation have been shown (132-141). However, the research on enterovirus and HLA class I is scarce. One group, has shown that pancreatic beta cells respond to coxsackievirus infection with upregulation of several HLA class I genes (247).

A number of genes with immunoregulatory properties increase the risk of several autoimmune conditions. Thus, it is hypothesized that some faulty immune regulation mechanisms might be shared by several diseases. In fact, amplified HLA class I has been shown in a number of autoimmune diseases, amongst them T1D and autoimmune hepatitis (248-250), as well as in systemic lupus erythematosus and autoimmune uveitis (251, 252).

HLA class I is normally expressed in all nucleated cells in the body. This fact makes our findings contradictory, since all cells, thyrocytes included, should have HLA class I expression. However, it is believed that a stable expression of HLA class I requires peptide loading (253). Moreover, we believe that there must be a certain amount of HLA class I expression before there is a positive immunostaining signal. Although, as previously discussed, IHC is not suitable for protein quantification.

Enteroviral capsid protein VP1

Enteroviral capsid VP1 is one of four capsid proteins shared by a range of enteroviral species (206). We found evidence of enterovirus in AITD samples, as well as in controls. The detection rate was significantly higher in GD samples than in controls. In HT patients however, this difference did not reach statistical significance. We herein actually replicate similar findings from the same cohort done in two previous studies; VP1 was found in both the HT and the GD group (204, 213). The same VP1 antibody (5D/8 clone from Dako®) was used, although with slightly different protocols and dilution. In addition, RNA in situ hybridization for enterovirus RNA was done in the two previous studies, and the results were comparable to the IHC results.

The enterovirus and AITD relationship has been examined before. The susceptibility of several enterovirus in thyroid cell lines was shown as early as the late 1950s (254, 255). As far as we know, the interest in examining enteroviral infections and thyroid disease did not re-emerge until several decades later, when an association with coxsackievirus was shown in case reports of toxic goiters and subacute thyroiditis (256, 257). Moreover, maternal enterovirus infection increases the likelihood of childhood and adolescent AITD (205). Desailloud et al investigated a collection of 86 thyroid tissue specimens derived from patients undergoing thyroid surgery for various reasons (202). Altogether, enterovirus RNA was detected in 25% of the patients, and no difference between patients with or without thyroid autoimmunity was found.

Not enough studies on enterovirus and AITD have been made to establish a causative relation. A reasonable conclusion, however, is that enterovirus is frequently found in thyroid tissue from patients with thyroid disease as well as in healthy individuals. Furthermore, we assume that enterovirus infection of the thyroid gland is common in the general population. In genetic susceptible individuals, enterovirus infection might contribute to initiate or sustain AITD.

Newly diagnosed GD vs chronic GD

Approximately 50% of patients with GD experience relapses, the reason for which is unknown. In our results we show some differences in the two patient groups. However, the study design (cross-sectional study) is not truly fit to answer this question, since some of the new GDs will develop chronic GD.

Interestingly, we found more VP1 in chronic GDs than in recent-onset GDs. With the limited methods applied in this study, we cannot know if the VP1 represents an active or latent enterovirus infection. However, we previously looked at enterovirus RNA with in situ hybridization in the same thyroid tissue samples, and found enterovirus RNA expression in 20% of GD samples, and in none of the controls (204). There was no significant difference in enterovirus RNA expression between recent-onset and chronic GDs.

In spite of our methods being unsuitable to answer these questions, we allow ourselves to speculate that the enterovirus seen in the chronic GDs represents a low-grade, persistent infection. Several studies have shown that enterovirus can persist in cells after primary infection (258, 259). Moreover, additional differences between the recent-onset GDs and chronic GDs previously shown in the same thyroid tissue collection, point to an active, antiviral immune response in new GD. New GD samples had a high density of plasmacytoid dendritic cells and expression of the IFNα downstream protein myxovirus

resistance protein 1 which indicate an active antiviral immune response (201). The CD8+ T cells were much more frequent in chronic GDs than in new GDs. This finding underpins our findings of more VP1 and HLA class I in chronic GDs. HLA class I presents viral fragments to CD8+ T cells, and viral infection attracts CD8+ T cells. Distinctions in the host's ability to clear virus might (in addition to other genetic and environmental factors such as smoking) contribute to protracted and relapsing disease in some individuals. In other words, persistent enterovirus infection in thyroid tissue contributes to chronic disease. As well as applying other methods, this hypothesis should be addressed with a different study design, allowing thyroid tissue biopsies in a study population at several time points.

STAT1 and PKR in AITD

Signal transducer and activator of transcription 1 and PKR are an essential antiviral protein and enzyme, respectively. The IHC method is flawed in both sensitivity and specificity. Therefore, we wished to support the VP1 findings with antiviral pathway proteins. We found STAT1 and PKR in a majority of the subset of tissue samples analyzed sequentially for HLA class I and STAT1 and PKR and VP1 (S.Table 3). HLA class I was colocalized with STAT1 and VP1 was colocalized with PKR within thyrocytes. Our results point to an activated antiviral pathway within thyrocytes.

The association of VP1 with an antiviral enzyme indicates that the VP1 immunopositivity represents an enteroviral infection, which has triggered an antiviral pathway. As already shown, enterovirus (more specifically coxsackievirus B5 and B3) induce gene expression of PKR, antiviral cytokines, STAT1 and several HLA class I genes (247, 260). Interestingly, PKR has also been found in association with VP1 in beta cells of patients with T1D (200). The fact that HLA class I was found in the same thyroid cells as STAT1 also indicates an active immune response. In viral infections, IFNs are produced as an important signaling protein. Upregulation of MHC class I is one of the consequences of increased IFN, and it has been shown that STAT1 is obligate in upregulation of MHC class I in response to IFNα or IFNγ (261). Our results support these findings, as HLA class I was found in the same thyroid cells as STAT1.

Some additional research, however scarce, supports our findings. Aphtovirus, which belongs, in addition to enterovirus, to the *Picornaviridae* family, caused upregulation of STAT1 in a bovine thyroid cell line (262). Moreover, transfection of a thyroid cell line with the viral products dsRNA and dsDNA caused upregulation of several antiviral signaling proteins and enzymes, including PKR, STAT and MHC class I (in addition to TLR3, ICAM-1, MAPK and NFkB) (263). STAT1 gain of function mutations were first described in 2011 in patients with chronic mucocutaneous candidiasis (264, 265). Interestingly, both

AITD (22%) and systemic viral infections (38%) are significantly more frequent in patients with STAT1 gain of function mutations than in the general population (266).

Common viruses are prevalent in the thyroid gland

We detected one or more viruses in a surprisingly high proportion of thyroid samples in paper 3, which is novel knowledge that adds evidence to the viral trigger hypothesis. Few other studies have looked at this wide range of viruses at once. However, one study investigated the presence of several herpes virus simultaneously in 22 AITD patients and found viral DNA in the majority of cases (175).

Autoimmune thyroid diseases are highly common; thus, an environmental triggering factor is expected to be widespread as well. The equal distribution in virus detection among AITD patients and controls can be explained by the strong genetic component in AITD pathogenesis. Patients with a known genetic predisposition for AITD increased their expression of another AITD susceptibility gene when exposed to products of microbial infection (267). This interplay of genetic predisposition and infectious insults could explain why common infections give rise to autoimmunity in some but are negligible in others.

Epstein-Barr virus has been implicated in MS for a long time. In a recent study with a population of 10 million people, a causal link was finally proved (268). This demonstrates that a seroprevalent virus can be implicated in the etiology of a rare disease.

Timing of infection may explain why frequent infections give rise to autoimmunity in some individuals. A recent study, for example, showed that EBV infection during early adulthood was highly associated with MS (269). It is biologically plausible that infections at particular times in life might apply for AITD as well. Especially since we know that there are peaks in AITD incidence in specific age groups.

A new paper exploring long-term symptoms after severe acute respiratory syndrome coronavirus 2 infection found that autoantibodies and reactivation of EBV were associated with a higher risk of long –term symptoms (270). The synergistic effect of EBV and HHV-6 in MS has also been proved (271).

Factors like timing of infection (for example in a time of hormonal changes as is seen in AITD incidence), genetic susceptibility, coinfections or reactivation of latent infections, may all contribute to the complex AITD etiology.

Causality and virus

Koch's Postulates:

1. The microorganism must be found in organisms suffering from the disease, but should not be found in healthy organisms.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy, susceptible host.
4. The microorganism must be reisolated from the inoculated, diseased host and identified as being identical to the original specific causative agent.

Koch's postulates, defined in the late 19th century, laid the premises for modern microbiology, in a time where the mere existence of microbes was controversial. Koch and his collaborator Loeffler, did not, however, write these postulates as they are known today, but rather they were derived from their work on anthrax and tuberculosis (272, 273). Even though microbial causality has been based on these postulates for almost two centuries, countless microbial diseases defy one or several of these postulates, as recognized by Koch himself when discovering asymptomatic carriers of *Vibrio Cholera*e (274). Moreover, the postulates were formulated before the discovery of virus, which cannot replicate in pure culture, thus defying the second postulate. However, in spite of not being applicable for many diseases of microbial origin, they served as a scientific set of rules, encouraging rigorous and reproducible methods. Subsequent critiques were fronted, especially after the discovery of virus (275). Falkow modified the postulates further, in line with the explosion in molecular biology techniques at the end of the 1980s (276).

We show evidence of several common viruses in AITD thyroid tissue and controls alike, which defies Koch's first postulate. The second, postulate, however is demonstrated in paper 3 where virus from thyroids replicated in cell cultures. The third and fourth postulates could be tested in AITD rat models (277).

Normally, virus cause disease by direct tissue damage, and resolve once the virus is cleared. Yet, persistent infections and chronic illnesses, due to both direct and indirect virus effect have been recognized due to extensive cancer research. The first human cancer virus was discovered in 1964, when EBV was discovered in a cell line from a Burkitt's lymphoma patient (278). However, it took nearly 30 years before EBV was officially recognized as a carcinogen. Traditional virological methods, such as viral culture and microscopy proved futile in consistently replicating the first findings. Moreover, the EBV

and cancer association defied Koch's postulates. That is, EBV infection was neither necessary nor sufficient for developing Burkitt's lymphoma.

Causality has been hard to prove in the viral infection-autoimmunity hypothesis as well. For various reasons, including innate properties of the virus, difficulties in discriminating between latent or active infection, challenges of virus detection remain. Moreover, infection might occur on such a modest scale, that it largely escapes detection. Fibrosis, tissue destruction and lymphoid displacement in thyroid tissue limit the possibility of finding virus even further. Finally, there might be a significant lag in the time of infection and debut of the disorder.

6 Main Conclusions

In this thesis I used IHC to investigate the presence of enterovirus and viral immune defense proteins in thyroid tissue from AITD patients and controls. In addition, a novel virus detection method using cell culture pre-enriched with thyroid tissue homogenates before PCR was applied in the search for multiple prevalent viruses.

HLA class I overexpression is a prominent feature in GD and HT alike, and supports the viral autoimmune trigger hypothesis due to the crucial antiviral properties of HLA class I. Moreover, the important antiviral proteins STAT1 and PKR are colocalized with HLA class I and VP1, respectively. This indicates an active, antiviral immune response within thyroid cells. Additionally, CAR is expressed in thyroid cells, thus demonstrating the presence of an enterovirus receptor.

In our search for several viruses in the thyroid gland, viral DNA or RNA could be found in the majority of the cases, and a large proportion of the samples were infected by multiple viruses. Few samples were completely devoid of viral RNA or DNA.

Viral infections and enterovirus in particular, seem to have a role in the AITD pathogenesis. Our methods for proving causality in complex diseases are, however, still too limited to provide conclusive evidence.

We argue that viral infection might initiate the autoimmune process in genetically susceptible individuals, but that the virus may be cleared or undetectable by the time of clinically significant disease.

7 Clinical Implications and Future Perspectives

7.1 Clinical Implications

The present studies demonstrate evidence of an active antiviral immune response in thyroid tissue from HT and GD patients. Several common viruses can infect the thyroid gland and are present in normal thyroid tissue as well as in thyroid tissue from AITD patients. Additionally, we shed light on important discrepancies between chronic and new GD patients.

Clinically, this knowledge can be used to explore antiviral treatment in newly diagnosed AITD patients. The results of ongoing trials with treatment of antivirals at T1D debut is awaited (279). If broad-spectrum antiviral treatment at an early stage of the disease can prolong insulin production in T1D, this might be applicable to HT as well (preserved or prolonged thyroid hormone production). Successful antiviral trials would add significant evidence to the viral infection hypothesis. Moreover, many viral infections are preventable with vaccines. In fact, one preclinical trial of a vaccine against coxsackievirus B prevented infection and T1D in mice, and the same vaccine is also highly immunogenic in primates (280).

Graves' disease has a high remission rate. Some clinical features predict a higher risk of recurrence, such as goiter size, younger age and high FT4 and TRAb values (281). Still, this is difficult to predict. For patients with adverse effect of antithyroid drugs, cardiac disease or planning pregnancies, recurring GD can be particularly challenging. These patients benefit from definitive treatment at an early stage. We found evidence of a persisting enteroviral infection in several chronic GDs. This difference could be exploited in the search for host factors that distinguishes the host's ability to clear enteroviral infection.

Autoimmune diseases affect a large percentage of the population, yet no cures exist and the treatments are merely disease modifying at best. Many autoimmune diseases share susceptibility genes; thus joint pathomechanistic features is a reasonable assumption. Hence, we believe our findings are generalizable to other autoimmune diseases.

7.2 Future Perspectives

The revolution of the microbiology, as well as epidemiology field in the 20th century enabled countless cause and effect discoveries. These findings led to the eradication of some diseases, and a radically decreased prevalence of others. The search for modifiable factors almost gained exclusive focus until the turn of the century. With the rapid development of genetics in the start of the 21st century, an expectation that many more diseases could be explained arose. Even though genetic susceptibility has been proved for most complex diseases, the missing heritability complex remain. In spite of GWAS from over 500 000 people, all susceptibility genes discovered only explain a fraction of the AITD prevalence. Traditional cause and effect models are based on the assumption that the cause has a strong effect, whereas for complex diseases several factors have, by themselves, a weak effect. This illustrates that more intricate models taking gene-gene interactions and gene-environment interactions into account must be applied in explaining the etiology of complex diseases. Dynamic feedback and changes over time add to the complexity. Furthermore, as illustrated by the EBV and cancer paradox, proving a viral association in multifactorial diseases is especially demanding. Finally, a more nuanced view of cause and effect, dividing it into predisposing factors (genes), enabling factors (stress, hormonal fluctuations, iodine status), precipitating factors (viral infections) and reinforcing factors (repeated or persistent viral infection) could be beneficial.

In this work, we present evidence of an association between viral infection of the thyroid and AITD. While enterovirus and AITD has been found previously, our finding of several common viruses at once in the thyroid gland is novel knowledge. This gives rise to questions such as low-grade persistent infections of common viruses in thyroid. But, it is important to emphasize that our findings need to be replicated in other thyroid sample collections, preferably with a larger control group with no thyroid disease whatsoever.

We do not know if the HLA class I found actually presents viral peptides or not, thus our study should be followed by HLA class I molecule complex characterization in thyrocytes, and its expression should be quantified by RNA studies. Moreover, it is now possible to describe the binding property of the HLA class I peptide binding groove. Furthermore, novel methods could be applied to characterize the specific T cell response in adaptive immunity (282). Finally, transcriptome analysis with next generation sequencing technology of infected thyroid cell lines should be done to solidify HLA class I findings.

Several enterovirus receptors are known. We provide evidence of CAR in thyroid cells. Other known enterovirus entry factors should be tested in thyroid cells. To test if other enterovirus than coxsackie- and adenovirus can exploit CAR is also of interest.

Enterovirus can be shed for a prolonged period of time from the upper respiratory and gastrointestinal tracts; up to four or 16 weeks, respectively (283). Hence, causality is hard to prove in these "permissive" sites. More studies are needed to prove whether or not the thyroid is a "permissive" enterovirus site.

As illustrated by the recent study that proved an association between EBV and MS (268), very large population data are needed to provide sturdy evidence of causation between a highly prevalent virus and a disease. Thyroid autoimmunity is, if judged by presence of thyroid antibodies alone, easy to define in large population biobanks, such as The Trøndelag Health Study and UK Biobank. An analysis combining thyroid antibody status in large populations with serological data is feasible. However, many viral infections cannot be diagnosed by serological methods. This has been the case for enterovirus in particular. Yet, promising serological methods which can detect multiple enteroviruses at once are emerging (284). Moreover, new methods using whole genome sequencing allows for discrimination of enterovirus serotypes (285). Additionally, full virome analyses are now available, which makes large population studies on all human viruses possible (286). This method, if applied on large populations could reveal whether or not some viral infections (or combinations of infections) are more prevalent in autoimmune patients.

In order to tease out cause and effect for prevalent viral infections, the experimental murine autoimmune thyroiditis model could be induced in axenic rats (free of all microorganisms) and rats exposed to various virus.

A potentially exciting method is to apply Mendelian randomization studies as well. Single nucleotide polymorphisms for specific viral infections have been detected, and their association with complex diseases have been done (287).

In all, steadily new technologies and research methods emerge and can be used in materials and tissue samples from patients with autoimmune diseases in order to find a cure, or even better, modifiable factors that prevent disease altogether. Nevertheless, this, as Rivers put it, *must be tempered by the priceless attributes of common sense, proper training and sound reasoning.*

Changes, notably the more extensive use of tissue-culture technics and serological reactions, will in the future undoubtedly occur in the methods of establishing the specific relation of viruses to disease; the number of changes will be limited only by the amount of ingenuity of investigators. To obtain the best results, however, this ingenuity must be tempered by the priceless attributes of common sense, proper training and sound reasoning.

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9 Appendix

Supplementary tables 1-4

Paper 1

Paper 2

Paper 3

Supplementary Table 1. Primary and secondary antibodies used in IHC and IF (papers 1 and 2)

Primary Antibody	Manufacturer and Clone	Antigen Retrieval	Antibody Dilution	Incubation Time with Primary Antibody	Secondary Detection System
VP1	Dako anti-enteroviral VP1 (5D8/1 clone)	10 mmol/l citrate pH 6.0	1/1400	30 minutes at room temperature	Dako REAL Envision Detection System (Agilent K5007)
HLA class I	Abcam Class I HLA Ab70328	10 mmol/l citrate pH 6.0	1/1500	1hr at room temperature	Dako REAL Envision Detection System (Agilent K5007)
CAR-SIV	Abcam100811	10 mmol/l citrate pH 6.0	1/1000	2hr at room temperature	Dako REAL Envision Detection System (Agilent K5007)
STAT1	Ab109320	10 mmol/l citrate pH 6.0	1/500	Overnight at 4°C	Immunofluorescence staining using anti-rabbit IgG (H+L) AlexaFluor ®488 conjugated secondary antibodies (1/400 for 1 hr) + DAPI (1/1000)
HLA class I	Abcam Class I HLA Ab70328	10 mmol/l citrate pH 6.0	1/1000	1hr at room temperature	Immunofluorescence staining using anti-mouse IgG (H+L) AlexaFluor ®555 conjugated secondary antibodies (1/400 for 1 hr)+ DAPI (1/1000)
VP1	Dako anti-enteroviral VP1 (5D8/1 clone)	10 mmol/l citrate pH 6.0	1/1000	Overnight at 4°C	Immunofluorescence staining using anti-mouse IgG (H+L) AlexaFluor ®488 conjugated secondary antibodies (1/400 for 1 hr)+ DAPI (1/1000)
PKR	Abcam Ab32052	10 mmol/l citrate pH 6.0	1/700	Overnight at 4°C	Immunofluorescence staining using anti-rabbit IgG (H+L) AlexaFluor ®555 conjugated secondary antibodies (1/400 for 1 hr)+ DAPI (1/1000)

Supplementary Table 2. List of cell lines, viruses, culture media, reagents, antibodies, instruments and databases used in paper 3.

Resource or reagent	Purchased from
Plasticware	
Flasks (T-25; T-75); 12-well and 6-well multiplates; cell scrapers; pipettes	Thermo Fisher Scientific-Corning (Monza, Italy)
Millicell EZ 4-well glass slides; Millex®syringe filter units, PVDF pore size 0.22 and 0.10 µm	Merck-Millipore (Vimodrone, Italy)
Cell culture	
Cell lines: AV3, RD, Ht-29, HEK293	European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK)
Cell line: VC3	American Type Culture Collection (ATCC, Manassas, VA)
Fetal bovine serum (FBS); DME/F12 medium with Hepes; L-Glutamine; Pyruvate; Penicillin-Streptomycin; Hank's balanced salt solution (HBSS); Dulbecco Phosphate-buffered saline (DPBS); Trypsin-EDTA	LifeTechnologies-Gibco (Monza, Italy)
Histopaque cell separation medium (density 1.077 and 1.119 g/ml); Bovine serum albumin (BSA); Collagenase type IV; Dispase-I	Sigma-Aldrich (Milano, Italy)
PANTA antibiotic mixture (Polymyxin B; Amphotericin B; Nalidixic Acid; Trimethoprin; Azlocillin)	BD (Milano, Italy)
MycoAlert Plus Mycoplasma Detection Kit; Accutase (cell detachment enzymes)	Euroclone-Lonza (Pero, Italy)
FastPrep-24 - Bead homogenizer for tissue	MP Biomedicals (Eschewege, Germany)
Molecular biology	
GoTaq DNA hot start DNA polymerase and master mix; DNA molecular weight markers; Tris-Acetate EDTA buffer (TAE); Agarose; High-resolution agarose	Promega Italia (Milano, Italy)
Reagents and disposables for the m2000sp automated instrument; DNA and RNA preparation kits; reagent vessels; deep well plates; disposable tips	Abbott Molecular (Rome, Italy)
Custom oligonucleotide primers; Whole Genome Amplification WGA-2 kit; Water (PCR-grade); Tris-EDTA buffer pH 8.0 (TE); low-EDTA (0.1 mM) TE buffer pH 8.0; Elution (10 mM Tris-Cl) buffer EB pH 8.5	Sigma-Aldrich (Milano, Italy)
Superscript III and Superscript IV reverse transcriptase with VILO master mix [containing ribonuclease inhibitor, helper and stabilizer proteins, random hexamer primers OR mixture of random hexamer primers plus oligo (dT)18; dNTPs; $MgCl_2$]; Platinum Taq hot start DNA polymerase and PCR Master Mix; Platinum GC Enhancer	Thermo Fisher Scientific-Invitrogen (Monza, Italy)
Brilliant II SYBR QPCR Master Mix with ROX passive reference dye	Agilent Technologies (Cernusco sul Naviglio, Italy)
GelRed stain	DBA Italia-Biotium (Segrate, Italy)
FlashGel - DNA electrophoresis screening system	Euroclone-Lonza (Pero, Italy)
ABI Prism: end-point Verity Dx thermal cyclers; real-time 7500 thermal cyclers. Gene sequencing: 3500 Genetic Analyzer.	Thermo Fisher Scientific (Monza, Italy)
BigDye Terminator V1.1 Cycle Sequencing Kit; Centri-Spin purification columns	Thermo Fisher Scientific-AppliedBiosystems (Monza, Italy)
LabChip GX Touch 24 Nucleic Acid Analyzer; HT DNA HS Reagents kit dual protocol; Hardshell PCR plate-96 blue	Perkin Elmer Italia (Milano, Italy)
Chemicals (molecular biology grade)	
Ethanol; Isopropanol; Dimethyl sulfoxide (DMSO); N,N-Dimethylformamide; Acetone; Paraformaldehyde 16% ampules (PFA); Triton X100; Tween-20; Na-Azide	Sigma-Aldrich (Milano, Italy)
Antibodies to enteroviruses and herpesvirus type 6; indirect immunofluorescence	
Mouse MAb to the panenterovirus antigen of the VP1 capsid protein, clone 9D5; mouse MAb to HHV-6 A & B envelope glycoprotein gp 60/110, clone	Merck-Millipore (Vimodrone, Italy)
Mouse MAb 5D-8.1 (panenterovirus directed to the VP1 capsid protein)	Dako (Milano, Italy)
Mouse MAb 6-E9/2 Magic (panenterovirus directed to the VP1 capsid protein)	Creative Diagnostics (Shirley, NY)
Mouse MAbs 3D-02 and 3D-05 (panenterovirus directed to the 3Dpol enzyme)	Our own laboratory
Mouse MAb to the HHV-6 capsid polypeptide 140K, clone F24H	Thermo Fisher Scientific (Monza, Italy)
Mouse MAb to the HHV-6 envelope glycoprotein 60/110 kDa	Santa Cruz Biortechnology - DBA Italia (Milano, Italy)
Antibodies to virus receptors (enteroviruses and herpesvirus type 6)	
Alexa Fluor 488 goat anti-mouse IgG; FITC goat anti-rabbit IgG; FITC rabbit anti-goat IgG; ProLong antifade; Evans Blue; DAPI	Thermo Fisher Scientific (Monza, Italy)
Rabbit Mab to CAR (coxsackie virus and adenovirus receptor) clone D3W3G; Rabbit polyclonal to DAF (decay-accelerating factor); Rabbit polyclonal to FCRN (neonatal Fc receptor, echoviruses-specific)	Thermo Fisher Scientific (Monza, Italy)
Rabbit antibody to PVR (CD155); Rabbit antibody to CAR; Rabbit antibody to DAF; Rabbit antibody to Integrin α2β1 (VLA2)	Cell Signaling - DBA Italia (Milano, Italy)
Rabbit antibody to SCARB2; Rabbit antibody to PSGL1; Rabbit antibody to Integrin alpha-V/beta-3 (ITGB3, CD62)	Antibody Genie - DBA Italia (Milano, Italy)
Rabbit antibody to Integrin alpha-V/beta-6 (KREMEN1); Rabbit antibody to CD134 (OX40); Mouse antibody MAb to CD46	Merck-Sigma (Milano, Italy)
Rabbit antibody to Heparan sulfate/Sialic acid; Mouse antibody MAb to ICAM-1 (1A29)	Thermo Fisher-Invitrogen (Monza, Italy)
Mouse MAb to ICAM-1 (CD54)	Novus Biologicals - DBA Italia (Milano, Italy)
Goat antibody to ICAM-5/Telencephalin	R&D Systems - BioTechne (Milano, Italy)
Mouse Mab to CD46 (Membrane cofactor protein) clone MEM-258	Merck-Sigma (Milano, Italy)
Mouse Mab to CD134 (OX-40, member of the TNF receptor superfamily) clone ACT35	Thermo Fisher Scientific (Monza, Italy)
Immunofluorescence microscopy	
Nikon E80i microscope with 10x, 20x, 40x IF objectives	Nikon (Firenze, Italy)
Applications and databases for virus sequences, statistics, graphics	
CLC Main Workbench	Qiagen (Milano, Italy)
https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/	NCBI Virus: viral sequence data from RefSeq, GenBank and other NCBI repositories
http://rvdb.dbi.udel.edu	Reference Viral Database (RVDB)
https://www.viprbrc.org/brc/home.spg?decorator=vipr	Virus Pathogen Resource, J. Craig Venter Institute
viralzone.expasy.org/	SIB Swiss Institute of Bioinformatics
GraphPad Prism 8	GraphPad Software, La Jolla, CA
Photoshop 22	Adobe, San Jose, CA

Supplementary Table 3. Oligonucleotide primer pairs used in end-point PCR for detecting enterovirus and HHV-6.

Oligo Name	Sequence 5' to 3'
EV5UTR-Tok-F	TCCTCCGGCCCCTGAATGCGGCTAATCC
EV5UTR-Tok-Rev	GAAACACGGWCACCAAAGTASTCG
EV5UTR-A-F	GTGTAGATCAGGTCGATGAGTCAC
EV5UTR-A-Rev	ATTGTCACCATAAGCAGCCA
EV5UTR-B-F	GACCAAGCACTTCTGTTACCC
EV5UTR-B-Rev	GTCACCATAAGCAGCCAATATA
EV5UTR-C-F	GGTGTGAAGAGCCTATTGAGC
EV5UTR-C-Rev	GATTGTCACCATAAGCAGCCA
EV5UTR-D-F	TGGTCCAGGCTGCGTT
EV5UTR-D-Rev	AACACGGACACCCAAAGTAGT
HHV-6-F [1]	GACAATCACATGCCTGGATAATG
HHV-6-Rev [1]	TGTAAGCGTGTGGTAATGGACTAA
1. With reference to sequence of the HHV-6 strain 02-572-M, the HHV-6 primers target nt 102772-102947 comprising partial sequences of the following genes: cytoplasmic envelopment protein 2 (CEP2) - tripartite terminase subunit 3 (TRM3). Amplicon size 175 nt.	

Supplementary Table 4. Commercial Real-Time PCR kits for detecting multiple viral agents

Description	Purchased from
Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Hepatitis C virus (HCV)	Abbott Molecular (Rome, Italy)
Parvovirus B19, Astrovirus species 1-8, Norovirus genogroup I and II, Rotaviruses	Altona Diagnostics (Segrate, Italy)
Sapoviruses	Creative Biogene (DBA, Milano, Italy)
Enteric Adenoviruses F40, F41	BD (Milano, Italy)

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Clinical Research Article

Clinical Research Article

HLA Class I Upregulation and Antiviral Immune Responses in Graves Disease

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Abbreviations: AITD, autoimmune thyroid disease; FFPE, formalin-fixed, paraffin-embedded; GD, Graves disease; GO, Graves ophthalmopathy; HLA, human leukocyte antigen; IF, immunofluorescence; IHC, immunohistochemistry; MxA, myxovirus resistance protein A; PDC, plasmacytoid dendritic cell; PKR, protein kinase R; STAT1, signal transducer and activator of transcription 1; TPO, thyroid peroxidase; TRAb, thyrotropin receptor antibodies; TSH, thyrotropin; VP1, enteroviral capsid protein 1.

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Abstract

Context: The origin of Graves disease (GD) remains elusive. However, evidence of an association between GD and viral infections is emerging. Human leukocyte antigen (HLA) class I presents viral antigens to circulating immune cells and plays a crucial role in the defense against viral infections.

Objective: This work aimed to investigate HLA class I expression, enterovirus presence, and the viral immune response proteins signal transducer and activation of transcription 1 (STAT1) and protein kinase R (PKR) in thyroid tissue from GD patients.

Methods: We collected thyroid tissue from core needle biopsies or surgical specimens from 48 GD patients and 24 controls. Standard immunohistochemistry was used to detect HLA class I and enteroviral capsid protein 1 (VP1) on formalin-fixed and paraffin-embedded tissue. STAT1 and PKR were examined by combined immunofluorescence staining. HLA class I expression score was the main outcome measure.

Results: The HLA class I expression score, which takes both proportion and intensity of immunostaining into account, was significantly higher in GD patients (3.1 ± 3.3) than in controls (0.5 ± 0.9) ($P < .001$). Significantly more VP1 positive thyroid cells were found GD samples (50.1 ± 30.5%) than in controls (14.9 ± 10.5%) ($P < .001$). STAT1 and HLA class I were found within the same thyroid cells and PKR and VP1 were also colocalized within thyroid cells.

Conclusion: HLA class I is upregulated in GD and enterovirus protein is prevalent in thyroid tissue. The colocalization of HLA class I with STAT1 and VP1 with PKR indicates an antiviral tissue response. These findings support the concept of a link between viral infections and GD.

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Key Words: Graves disease, autoimmune thyroid disease, HLA class I, STAT1, enterovirus, viral infections

Graves disease (GD) is a prevalent, autoimmune endocrine disease, caused by an interplay between genetic and environmental factors (1, 2). The incidence of GD shows seasonal trends and variable geographical distribution, indicating possible epidemic triggering factors (3). Evidence of an association between viral infections and autoimmune thyroid disease (AITD) is emerging (4-11). Enterovirus, in particular, has been linked to numerous autoimmune diseases, and the association with type 1 diabetes is especially strong (12-15).

The class I major histocompatibility complex, usually known as human leukocyte antigen (HLA) class I in humans, is a complex of molecules expressed on nucleated cells. HLA class I normally presents peptides from intracellular host proteins. During viral infections, HLA class I presents viral antigens on the cell surface, which is vital for recognition and destruction of infected cells by $CD8^+$ T cells (cytotoxic T cells). By contrast, HLA class II presents with extracellular pathogens, such as bacteria.

Antigen presentation is possibly a key process in inducing thyroid autoimmunity. An association between aberrant HLA class II expression and thyroid autoimmunity is recognized (16), and the HLA class II gene variant *HLA-DR3*is a major AITD susceptibility gene (17-19). HLA class I and its role in thyroid autoimmunity, on the other hand, is less explored. However, HLA class I alleles have been linked to AITD (20, 21), and our group recently reported that HLA class I is upregulated in Hashimoto thyroiditis (22).

HLA class I is upregulated in response to interferon-activated signal transducer and activator of transcription 1 (STAT1) (23). Moreover, STAT1 promotes transcription of several genes involved in antiviral defense mechanisms, one of which is protein kinase R (PKR) (24-30), an enzyme that inhibits viral protein synthesis (31, 32).

To further investigate the association between viral infection and thyroid autoimmunity, we aimed to i) examine the expression of HLA class I in thyroid cells in GD, ii) examine the presence of the antiviral immune response proteins STAT1 and PKR in GD, and iii) confirm the presence of enterovirus in GD. Using immunohistochemistry (IHC) and combined immunofluorescence (IF), we evaluated the expression of HLA class I, STAT1, PKR, and enteroviral capsid protein 1 (VP1) in thyroid tissue from a cohort of both recent-onset and chronic GD.

Material and Methods

Study participants and thyroid tissue collection

For this study, we used previously collected thyroid tissue samples from 48 patients with GD and 24 controls at Oslo University Hospital (11). Age, sex, smoking habits, thyroid function, disease duration, and treatment at the time of tissue sampling were recorded (Table 1). The duration of disease was defined as the time elapsed between clinical diagnosis and the collection of thyroid tissue. The patients were divided into 2 subgroups according to disease duration: newly diagnosed GD (within the last 3 months) or chronic GD (median disease duration of 24 months). Thyroid tissue samples were collected by core needle biopsy in patients with newly diagnosed disease, and from surgical specimens during thyroidectomy in patients with chronic disease. Thyroid tissue samples from 24 patients undergoing neck surgery for reasons other than AITD, such as thyroid tumors or parathyroid adenomas, served as controls. Control samples were taken from normal thyroid tissue adjacent to the pathological lesion. To insure no preexisting or unrecognized thyroid autoimmunity, we measured serum antibodies against thyroid peroxidase (TPO-Ab), thyroglobulin, and thyrotropin receptor (TRAb) in all control individuals.

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were cut into 3-µm slices and mounted on slides for further analysis. The regional ethics committee approved the study and written informed consent was obtained from all participants.

Immunohistochemistry

HLA class I and VP1 immunostaining was performed with a standard IHC protocol in all samples. Antigens were unmasked by heating in 10-mM citrate buffer of pH 6.0, in a pressure cooker in a microwave oven (800 W) for 20 minutes, followed by 20 minutes of cooling at room temperature. Sections were blocked with 10% goat serum and primary antibodies diluted in Dako REAL antibody diluent (Agilent S202230). The Dako antienteroviral VP1 (5D8/1 clone) was used at a dilution of 1:1400 (55 ng/mL) for 30 minutes. The HLA class I primary antibody (Abcam class I HLA [EMR8/5] Ab70328) was used at a dilution of 1:1500 for 1 hour. Primary antibodies were visualized using the Dako REAL EnVision Detection system (Agilent K5007).

Combined immunofluorescence

To examine the presence of STAT1 and PKR, and to test the localization of these proteins in relation to HLA class I and VP1 within the same FFPE sections, we performed combined IF of STAT1/HLA class I and PKR/VP1. Owing to limited resources and a restricted supply of the STAT1

Table 1. Characteristics of Patients and Controls at Study Inclusion

	Control group	Graves disease, all	P^a	New Graves disease	Chronic Graves disease	P^b
	(n = 24)	(n = 48)		(n = 22)	(n = 26)	
Duration, mo	–	18.0 ± 28.0	–	0.0 (2.4)	24.0 (114)	–
Age, y	52.0 ± 14.4	44.0 ± 13.1	.021	40.1 ± 11.9	47.2 ± 13.4	.062
Female, No., %	22 (91.7)	42 (87.5)	.596	19 (86.4)	23 (88.5)	.828
TSH, mIU/L	1.4 (0.7-2.5)	0.0 (0.0-0.0)	< .001	0.0 (0.0-0.0)	0.0 (0.0-0.3)	.003
FT4, pmol/L	13.1 (12.1-14.5)	24.2 (16.7-45.8)	< .001	40.0 (27.0-55.2)	18.6 (15.6-21.4)	< .001
TRAb, IU/L	< .09	8.6 (3.4-16.6)	< .001	8.6 (4.7-12.1)	8.9 (2.7-18.0)	.796
Smoker, No., %	5 (21.7)	18 (40.9)	.119	5 (25.0)	13 (54.2)	.053
Ophthalmopathy, No., %	0 (0.0)	23 (50.0)	< .001	3 (15.0)	20 (76.9)	< .001
Antithyroid drugs, No., %	0 (0.0)	23 (47.9)	< .001	0 (0.0)	23 (88.5)	< .001
Steroids before inclusion, No., %	0 (0.0)	10 (20.8)	.017	0 (0.0)	10 (38.5)	.001

Values presented as median and range, median and interquartile ranges, mean±SD, numbers and percentages. Reference ranges: TSH equal to 0.5 to 3.6 mIU/L, FT4 equal to 8 to 20 pmol/L, TRAb less than 1.5 IU/L. *P* values compared to controls and tested with *t* test, Mann-Whitney *U* test, or Pearson chi-square test. Smoking and ophthalmopathy data were missing for 7 patients.

Abbreviations: FT4, free thyroxine; TRAb, thyrotropin receptor antibody; TSH, thyrotropin.

[a]P controls vs all Graves patients. [b]P newly diagnosed vs chronic disease.

antibody, we performed IF staining in a subset of the samples only. Seventeen samples of especially good quality and size were analyzed. Antigen retrieval and blocking were performed as described in the IHC protocol described earlier. The sections were immunostained sequentially with STAT1 (Ab109320, dilution 1:500, overnight primary incubation at 4 °C), followed by HLA class I (Abcam class I HLA [EMR8/5] Ab70328, dilution 1:1000, 1 hour at room temperature). The same subset of cases were stained for PKR (Abcam Ab32052 at a dilution of 1:700, overnight at 4 °C) and VP1 (Dako antienteroviral VP1 [5D8/1 clone], 1:1000 dilution, overnight at 4 °C). Primary antibodies were detected using species-specific secondary antibodies (antirabbit or antimouse immunoglobulin G [H + L]) conjugated to either Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen) at a dilution of 1:400 for 1 hour at room temperature, together with DAPI 1:1000.

Image acquisition and analysis

Bright-field image acquisition and analysis were performed on a Nikon 50i microscope fitted with a DS-Fi camera and a DSL2 camera control unit. Images were captured and analyzed using the ImageJ platform. IF image collection and processing were achieved using a Leica DM4000 B LED upright fluorescence microscope and Leica Image analysis software (LAS X).

Immunohistochemical analysis

The slides were analyzed by light microscopy at 400× magnification by 2 independent scientists (T.W. and S.J.R.). Tissue samples were interpreted in a blinded fashion. First, samples were classified as positive or negative for HLA class I. Only immunostaining of thyroid follicular cells (thyrocytes) was evaluated. Next, HLA class I immunoreactivity was graded according to the semiquantitative Allred scoring system. The Allred scoring system takes both intensity (ranging from 0 to 3) and proportion (ranging from 0 to 5) into account, with 8 being the maximum score possible and 0 being the lowest score. This system is commonly used in clinical settings to assess the immunostaining of pathological specimens (33).

We assessed VP1 staining by counting all positively stained thyrocytes alongside the total number of thyrocytes in 10 consecutive counting grids (0.058 mm^2) at 400× magnification, thus yielding a percentage of positively stained thyrocytes.

Statistics

If normally distributed, continuous variables were summarized with means and SD (mean ± SD) and, if skewed, with medians

and interquartile ranges (median [25th percentile to 75th percentile]). Categorical variables were summarized as frequencies and percentages. Statistical significance of differences between groups was calculated with the independent-samples *t* test if fulfilling criteria of normality, and with the Mann-Whitney *U* test if not. The Pearson chi-square test was used to determine the statistical significance of differences in proportions. Associations between clinical features and results were explored using binary logistic regression, Pearson correlation for continuous data, and the Spearman correlation for ordinal data. All analyses were performed using IBM SPSS Statistics version 25 and GraphPad Prism 7.02. We considered *P* values less than .05 significant.

Results

Few studies have looked at the immunological tissue responses in GD thyroid tissue. In the present study, we found HLA class I upregulation and antiviral proteins both in recent-onset GD and chronic GD.

Study population

Thyroid samples from 26 patients with chronic GD, 22 patients with newly diagnosed GD, and 24 controls were analyzed. Patient characteristics and demographic data are presented in Table 1. All new GD samples were collected within 3 months after diagnosis, while the median disease duration before sampling was 24 months for chronic GD. None of the recent-onset GD patients received antithyroid treatment prior to inclusion, whereas 23 out of 26 chronic GD patients were on antithyroid treatment (22 on carbimazole and 1 on propylthiouracil) at inclusion. The GD group was significantly younger (44.0 ± 13.1 years) than the control group (52.0 ± 14.4 years). Eighteen out of 44 (40.9%) GD patients and 5 out of 23 (21.7%) controls were current cigarette smokers (*P* = .119). Smoking status data was missing from four GD patients and one control. The sex distribution was equal in each group (87.5% female in GD and 91.7% female in the control group). Three patients in the GD group (6.3%) had one additional autoimmune disease (ankylosing spondylitis, Addison disease and Sjögren syndrome). Two patients in the control group (8.3%) had an autoimmune disease (ulcerous colitis and psoriasis). Ten chronic GD patients received steroids (methylprednisolone intravenously n = 6, prednisolone orally n = 4) due to Graves ophthalmopathy (GO).

Human leukocyte antigen class I is upregulated in Graves disease

HLA class I presents viral antigens to $CD8^+$ T cells of the immune system and plays a vital role in the body's defense against virus. We herein demonstrate that HLA class I expression is significantly higher in GD compared to controls (Fig. 1A). First, we examined the number of positive samples in GD and controls. We found increased HLA class I expression in thyroid tissue in 25 of 48 (52.1%) samples in the GD group, but in only 5 of 24 (20.8%) samples in the control group (*P* = .011). The chronic GD group had a significantly higher number of HLA class I–positive samples than controls, with 15 of 26 (57.7%) being positive (*P* = .012) (Fig. 2A). The proportion of HLA class I–positive samples in the new GD group was also higher than in the control group (45.5% vs 20.8% respectively); however, the difference did not reach statistical significance. The proportion of HLA class I–positive samples did not differ significantly between the recent-onset and chronic GD group.

Next, we assessed HLA class I expression using an IHC scoring tool that takes both intensity and proportion of immunostaining into account (ranging from 0 to 8) (33). The mean HLA class I expression score was 3.1 ± 3.3 in GD patients and 0.5 ± 0.9 in controls (*P* < .001). Each subgroup had a significantly higher HLA class I expression score compared to the control group (3.4 ± 3.3 in chronic GD and 2.8 ± 3.3 in new GD) (Fig. 2B). Additionally, when considering HLA class I–positive samples only (score ≥ 1), the HLA class I expression score differed significantly between GD patients (6.0 ± 1.7) and controls (2.2 ± 0.4) (*P* < .001). Moreover, no samples in the control group had an HLA class I expression score higher than 3. The HLA class I expression score did not significantly differ between the two GD subgroups.

The proportion of HLA class I–positive samples was slightly higher in the 10 patients who received steroids before study inclusion (60.0%) compared to the group that did not receive steroids (50.0%) (*P* = .578). Moreover, the HLA class I score was higher (4.0 ± 3.6) in the steroid group compared to the GD patients who did not receive steroids (2.9 ± 3.2) (*P* = 0.347).

Thyroid function (thyrotropin [TSH] and free thyroxine), TRAb level, age, smoking status, and duration of disease did not significantly influence or correlate with the proportion of HLA class I–positive samples or HLA class I expression score. Although the mean TRAb was higher in the HLA class I–positive samples (17.7 ± 5.8 IU/L) than in the HLA class I–negative sample (9.3 ± 1.5 IU/L), the difference did not reach statistical significance.

Smoking increases the risk of GD, and especially GO, but several studies indicate that smoking protects against the development of TPO-Ab and thyroglobulin antibodies (34-37). In the present study, the proportion of GD patients with positive TPO-Ab was not different between smokers and nonsmokers (68.4% vs 66.7%, respectively) (*P* = .907). The median TPO-Ab titer, however, was nonsignificantly lower in smokers compared to nonsmokers (124 vs 180 kIU/L) (*P* = .511). Smokers had a higher median thyroglobulin antibody titer than nonsmokers (742 vs 116 kIU/l) (*P* = .356).

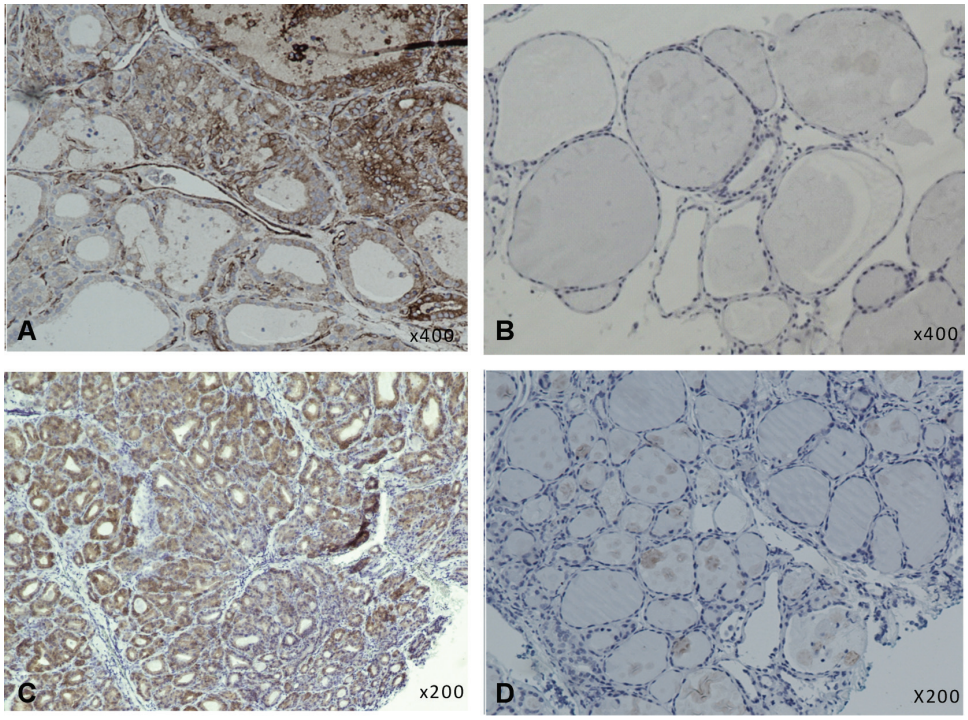

Figure 1. Immunohistochemical staining for HLA class I and VP1 in thyroid tissue samples from GD patients and controls. HLA class I and VP1 was found in more GD samples than controls. A, Positive HLA class I staining (brown color) in thyroid follicular cells in a new GD sample. B, Negative HLA class I staining in a control sample. C, Globally positive VP1 staining (brown color) in thyroid follicular cells in a chronic GD sample. D, Negative VP1 staining in a control sample. All samples stained with a standard horseradish peroxidase immunohistochemistry protocol. GD, Graves disease; HLA, human leukocyte antigen; VP1, enteroviral capsid protein 1.

Signal transducer and activation of transcription 1 is colocalized with human leukocyte antigen class I within thyroid cells

STAT1 is a cytoplasmic protein, which on activation by interferons translocates to the nucleus and initiates transcription of antiviral response proteins. We analyzed STAT1 in relation to HLA class I expression in a subset of samples (4 with newly diagnosed GD, 9 chronic GD, and 4 controls). Interestingly, we found elevated STAT1 in all 4 GD samples with newly diagnosed disease and in only 3 of 9 samples in the chronic GD group. Two of the 4 control samples were also positive (Table 2). STAT1 expression was colocalized with HLA class I, and we observed both cytosolic and nuclear STAT1 (Fig. 3A).

Enteroviral capsid protein 1 was detected in thyroid tissue

The presence of enterovirus can be explored by staining for the enteroviral capsid protein VP1 (38) (Figs. 1C and 3C). We previously reported both increased VP1 and the presence of enterovirus RNA (in situ hybridization) in the same GD cohort (11). VP1 was found in more GD samples (29 of 48 [60.4%]) than in controls (10 of 24 [41.7%]). This difference was significant only when comparing chronic GD patients (19 of 26 [73.1%]) to control individuals (10 of 24 [41.7%]) ($P = .025$). When analyzing the VP1-positive samples, there were also significantly more $VP1^+$ thyrocytes in the GD samples (median 40.0% [19.0%-80.9]) than in the controls (median 11.7% [7.7%-18.1%]) ($P < .001$) (Fig. 4A). The 2 disease stages differed in the numbers of $VP1^+$ thyrocytes, with the chronic GD patients having a significantly higher number (median 70.4% [33.2%-91.5%]) than the newly diagnosed GD group (median 19.0% [16.3-35.4%]) ($P = .003$) (Fig. 4A). Finally, significantly more GD samples (20 out of 48 [41.7%]) than control samples (2 out of 24 [8.3%]) ($P = .004$) were double positive (both $VP1^+$ and HLA class I^+ thyrocytes) (Fig. 4B).

VP1 and HLA class I expression score were positively correlated (Pearson correlation = 0.48, $P < .001$). Moreover, the odds of being HLA class I positive were 4.0 times higher in the VP1-positive group compared to the VP1-negative group ($P = .007$).

Protein kinase R was colocalized with enteroviral capsid protein 1

The antiviral enzyme PKR inhibits viral messenger RNA translation, thus preventing viral replication. The transcription of PKR is positively regulated by STAT1. We detected PKR in 11

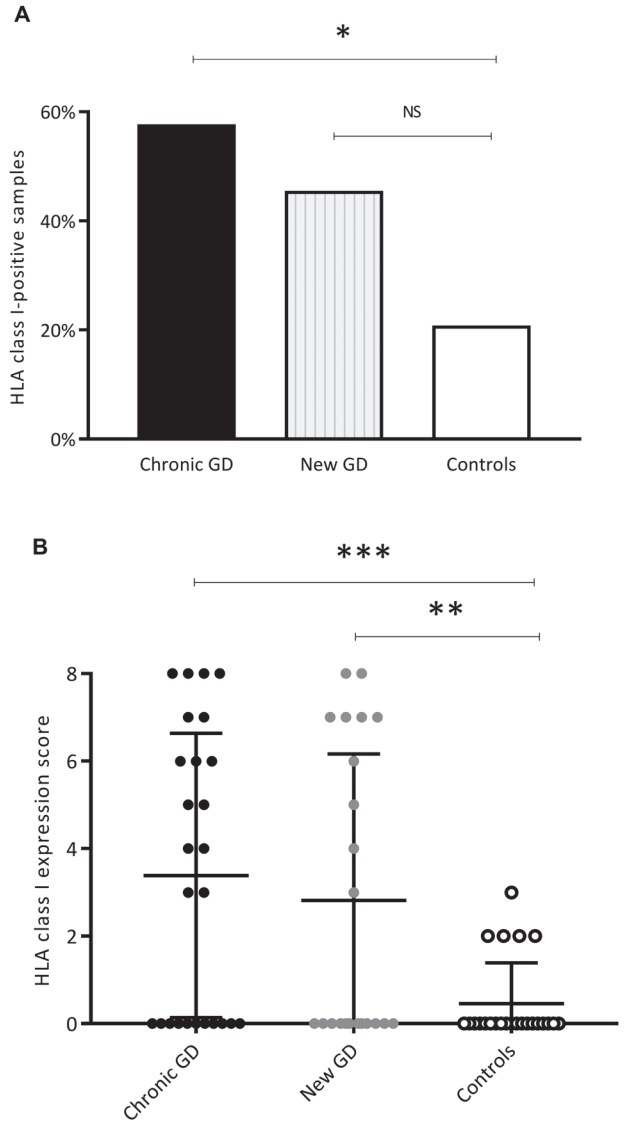

Figure 2. HLA class I immunodetection and HLA class I expression score. Significantly more HLA class I–positive samples were found in the GD group than in the control group. A, Proportion of samples with HLA class I positivity in the 2 GD subgroups and in the controls. B, HLA class I expression score (Allred score), taking both immunostaining intensity and proportion of stained tissue into account (maximum score 8, and lowest score 0). The HLA class I expression score was significantly higher in the chronic GD group and in the newly diagnosed GD group than in controls. Bars represent mean and SD. **P* less than or equal to .05; ***P* less than or equal to .01; *** *P* less than or equal to .001. GD, Graves disease; HLA, human leukocyte antigen; NS, not significant.

out of 13 GD samples and in 1 out of 4 control samples (see Table 2). PKR was found in all new GD samples. Interestingly, PKR was colocalized with VP1 within the same thyroid cells (see Fig. 3C).

Plasmacytoid dendritic cells correlate with human leukocyte antigen class I

We previously reported an increased density of plasmacytoid dendritic cells (PDCs) and upregulated expression of the type 1 interferon downstream response protein myxovirus resistance protein A (MxA) in newly diagnosed GD (39). $CD8^+$ T cells, on the other hand, were mainly found in chronic GD in this cohort (39). $CD8^+$ T cells recognize the HLA class I–viral antigen complex, and induce destruction of virally infected cells. When combining previous results with current HLA class I findings, we found a significantly positive correlation between HLA class I score and density of number of PDCs in thyroid tissue (Pearson correlation 0.332, $P = .029$). The number of $VP1^+$ thyrocytes correlated with $CD8^+$ T cells (Pearson correlation = 0.338, $P = .012$). Ten of 25 (40.0%) VP1-positive GD samples had $CD8^+$ T cells, whereas only one of the 16 (6.3%) VP1-negative GD samples had $CD8^+$ T cells ($P = .017$).

Discussion

In this study consisting of thyroid tissue samples both from newly diagnosed and chronic GD patients, we show that HLA class I upregulation is a prominent feature of GD. Moreover, HLA class I is colocalized with the antiviral immune response protein STAT1, whereas the enterovirus capsid protein VP1 is colocalized with PKR, suggesting an active antiviral thyroid tissue immune response in GD. Additionally, we confirm earlier findings of VP1 in the same cohort, which was particularly prevalent in chronic GD tissue samples.

HLA class I was upregulated in thyroid cells both in chronic and new GD tissue samples. These findings are in line with the seminal work of Hanafusa and Bottazzo, who found upregulated HLA class I in 16 out of 26 surgical thyroid samples from GD patients (16). We found increased HLA class I expression in 5 out of 24 controls, but the intensity and proportion of HLA class I staining were significantly lower in the control samples than in the GD samples.

Amplified HLA class I has been found in several autoimmune diseases, with type 1 diabetes being the most studied (40). The role of the amplified HLA class I expression is, however, unknown. We hypothesize that the amplified HLA class I response is due to an active immune response following viral infection. Numerous theories on how viral infections can induce autoimmunity exist (reviewed in [41]). Infection leads to the production of inflammatory cytokines and expression of costimulators on antigen-presenting cells, thus attracting immune cells to the infected area and decreasing the threshold for self-attack. Interferon α, which is produced in large quantities by PDCs in viral infections, stimulates HLA class I upregulation (42) and induces thyroglobulin degradation (43), which is a potential mechanism for pathological thyroglobulin presentation. Moreover, infectious microbes may produce

Table 2. Sequential immunofluorescence in a subset of samples

	HLA class I	STAT 1	PKR	VP1
Chronic GD 1	**Positive**	Negative	Negative	Negative
Chronic GD 2	**Positive**	**Positive**	**Positive**	**Positive**
Chronic GD 3	**Positive**	Negative	**Positive**	**Positive**
Chronic GD 4	Negative	Negative	**Positive**	**Positive**
Chronic GD 5	**Positive**	Negative	**Positive**	**Positive**
Chronic GD 6	**Positive**	**Positive**	**Positive**	**Positive**
Chronic GD 7	Negative	Negative	Negative	Negative
Chronic GD 8	Negative	Negative	**Positive**	**Positive**
Chronic GD 9	**Positive**	**Positive**	**Positive**	**Positive**
New GD 1	**Positive**	**Positive**	**Positive**	**Positive**
New GD 2	**Positive**	**Positive**	**Positive**	**Positive**
New GD 3	**Positive**	**Positive**	**Positive**	**Positive**
New GD 4	**Positive**	**Positive**	**Positive**	**Positive**
Control 1	**Positive**	**Positive**	**Positive**	**Positive**
Control 2	**Positive**	**Positive**	Negative	Negative
Control 3	Negative	Negative	Negative	Negative
Control 4	Negative	Negative	Negative	Negative

Seventeen samples were stained sequentially with immunofluorescence for HLA class I, STAT1, PKR, and VP1. The table shows that the majority of the GD samples were both HLA class I and STAT1 positive (shown in bold). All new GD samples stained positively for all proteins studied.
Abbreviations: GD, Graves disease; HLA, human leukocyte antigen; PKR, protein kinase R; STAT1, signal transducer and activator of transcription 1; VP1, enteroviral capsid protein 1.

antigens similar to self-antigens, thus evoking an immune attack against the self. Proteins derived from infectious agents, such as *Borrelia burgdorferi* and *Yersinia enterocolitica*, show structural homology with the TSH receptor (44). Furthermore, tissue injury might lead to the release of otherwise hidden antigens that can prompt an immune response. Finally, HLA class I upregulation might be a virus-evading strategy. HLA class I acts as a self-tolerance checkpoint for natural killer cells. Some viruses, including hepatitis C virus, herpesvirus, and flavivirus, induce upregulation of HLA class I to avoid natural killer cell–mediated attacks (45-47).

Antithyroid drugs and steroids both are immunosuppressive, and a suppressive effect on HLA class I cannot be ruled out. However, we could not find any significant differences in HLA expression in participants treated with steroids or antithyroid drugs compared to those without any treatment.

Interestingly, Mack et al showed that orbital fibroblasts from GD patients expressed HLA class II in response to cigarette toxins in combination with interferon γ stimulation (48). The increased capacity of antigen presentation due to smoking and interferon γ, which is a viral response cytokine, might be one of the reasons that smokers are more prone to develop GO. However, no such association has been shown for HLA class I expression and GO. We found more HLA class I–positive samples and a higher HLA class I score in smoking GD patients, but the difference did not reach statistical significance.

Viral infections have been linked to several autoimmune diseases, and there is evidence of persistent enterovirus infection of the β cells of the pancreas in patients with type 1 diabetes (12, 13). Enteroviruses, among other viruses, have also been associated with AITD (4-11). In patients genetically predisposed for AITD, an additional AITD-susceptibility gene was expressed when exposed to products of microbial infection (49). This interplay of genetic predisposition and infectious insults could explain why common infections give rise to autoimmunity in some but are negligible in others. To perform double staining for VP1 and antiviral tissue markers, we first repeated VP1 staining and found similar results as previously reported (11). Immunoreactive VP1 was present in thyroid samples from GD patients and controls, but significantly more VP1 was found in GD patients than in control individuals. Notably, the chronic GD tissue samples had remarkably high VP1 numbers. Moreover, the positive correlation between VP1 and HLA class I might indicate that the HLA class I upregulation is a response to enterovirus infection. Interestingly, we previously confirmed the presence of the SIV isoform of the coxsackie and adenovirus receptor in thyroid tissue, thus proving that thyroid cells are susceptible to enteroviral infection (22).

STAT1 was detected with an antiserum that does not discriminate between phosphorylated and nonphosphorylated STAT1. However, the active, phosphorylated form of STAT1 relocates from the cytosol to the nucleus. We identified

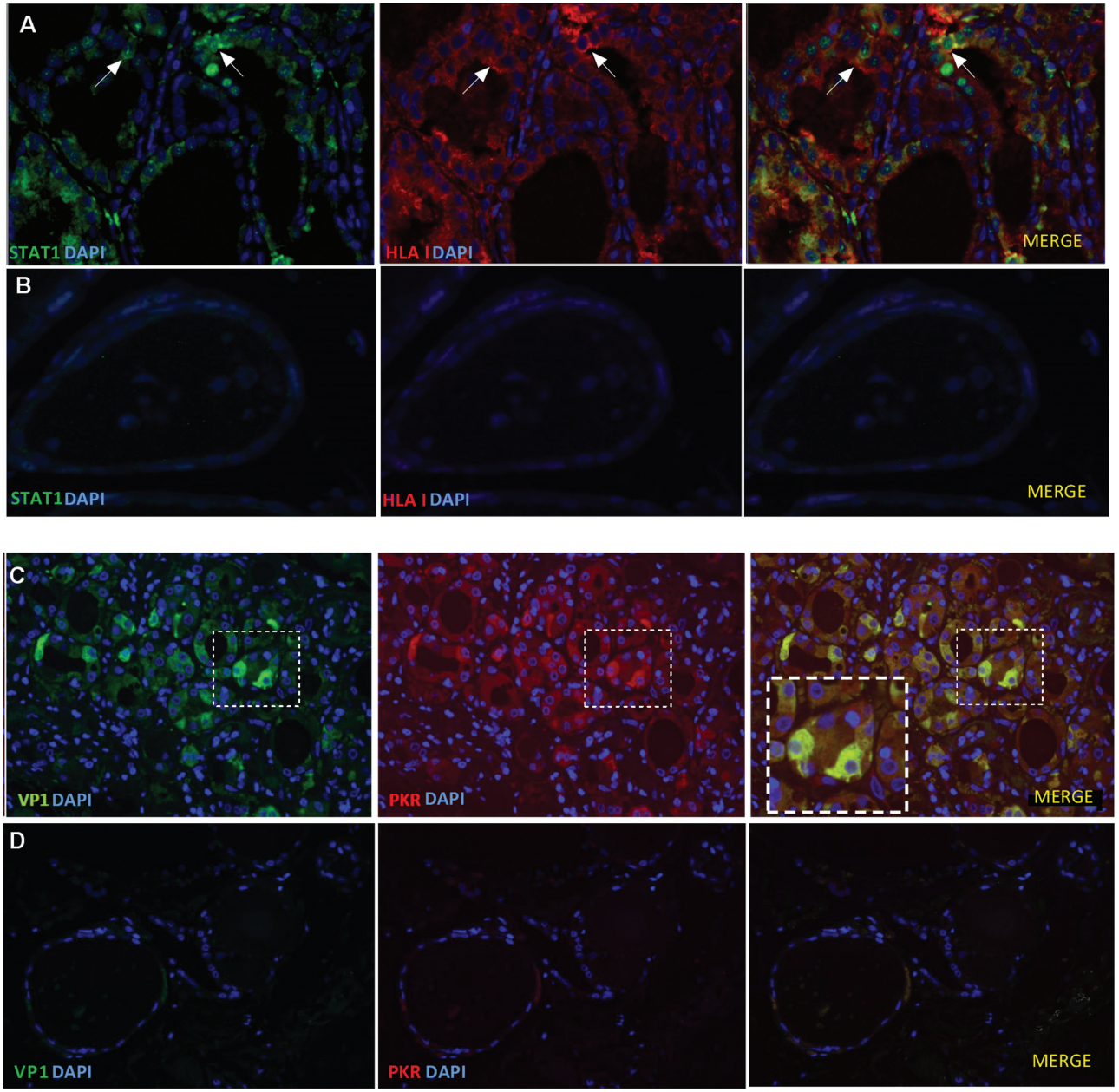

Figure 3. Combined immunofluorescence of HLA class I/STAT1 and PKR/VP1 in GD samples and controls. Nuclear and cytosolic STAT1 was found in thyroid cells and was colocalized with HLA class I. PKR and VP1 were also colocalized within thyroid cells. A, An example of STAT1 (green) and HLA class I (red) immunofluorescence staining in GD thyroid tissue. Arrows indicate thyroid cells with nuclear STAT1 and intracellular HLA class I. B, Control sample with negative STAT1 and HLA class I staining. C, An example of VP1 (green) and PKR (red) immunofluorescence staining in GD thyroid tissue. The insets represent higher magnification (of the area outlined by the white boxes) and shows colocalized VP1 and PKR within thyrocytes. D, Control sample with negative VP1 and PKR staining. Merged images with blue nuclear DAPI staining. All scale bars at 25 μm. DAPI, 4′,6-diamidino-2-phenylindole; GD, Graves disease; HLA, human leukocyte antigen; PKR, protein kinase R; STAT1, signal transducer and activator of transcription 1; VP1, enteroviral capsid protein 1.

both cytosolic and nuclear STAT1, suggesting the presence of both inactive and active forms of STAT1 (see Fig. 3A). We found that STAT1 colocalized with HLA class I, thus supporting the previously proven link between activated STAT1 and upregulated HLA class I (23). The antiviral protein PKR, which is enhanced by STAT1 activation, was also found in the GD samples. PKR was colocalized within the same thyroid cells as VP1, thus demonstrating that enterovirus infection may lead to PKR expression (see Fig. 3C). This response resembles that of studies in which infection of thyrocytes or stimulation of cells with viral products caused upregulation of HLA class I, PKR, STAT1, and toll-like receptor 3, among other immune proteins (4, 10, 50).

All recent-onset GD analyzed for STAT1 and PKR were positive, suggesting an active, interferon-driven immune

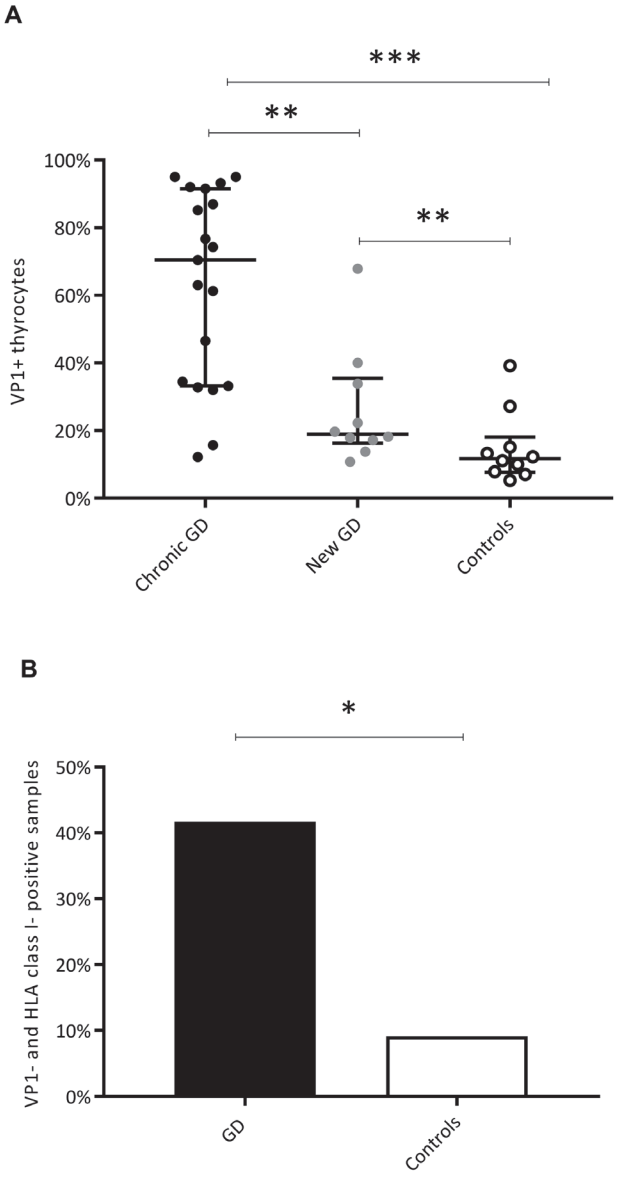

Figure 4. VP1 assessment in GD samples and controls. We confirmed the presence of VP1 in GD samples. A, Number of VP1$^+$ thyrocytes in the GD subgroups and controls. There were significantly more VP1$^+$ thyrocytes both in the chronic GD group and the new GD group compared to controls. The chronic GD group had significantly more VP1$^+$ thyrocytes than the new GD group. Bars represent median and interquartile range. B, Proportion of double-positive samples (VP1$^+$/HLA I$^+$) in the GD group and the controls. We found significantly more double-positive samples in the GD group than in the control group. *P less than or equal to .05; **P less than or equal to .01; ***P less than or equal to .001. GD, Graves disease; HLA, human leukocyte antigen; VP1, enteroviral capsid protein 1.

response. Moreover, we confirmed that the chronic GD participants had more VP1$^+$ thyroid cells. These findings are consistent with our previous results, which also showed an interferon-driven response in recent-onset GD. PDCs, which secrete type 1 interferon in the presence of virus, were prevalent in recent-onset GD tissue. Likewise, the type 1 interferon surrogate marker MxA was also prominent in recent-onset GD (39). CD8$^+$ T cells, on the other hand, were characteristic of chronic GD (39). When combining results from these previous analyses performed in the same cohort, our findings point to an acute antiviral response in recent-onset GD and a persistent viral infection in chronic GD.

We show some important differences between chronic and new GD, hypothesizing that these 2 patient groups might differ in their ability to resolve viral infection. However, our method of detecting enterovirus is not suitable to answer whether the VP1 represents persistent or acute infection. As well as applying other methods, this hypothesis should be addressed with a different study design, allowing thyroid tissue biopsies in a study population at several time points. Moreover, new technologies that allow us to target the peptide-binding capacities of the HLA molecules could provide answers as to what thyroid cells are presenting (51). Furthermore, novel methods could be applied to characterize the specific T-cell response in adaptive immunity (52). Finally, more sensitive methods than IHC should be applied to confirm the presence of virus in thyroid tissue in autoimmune thyroid diseases. Yet, the timing in break of tolerance and debut of clinical manifestations in autoimmune diseases are difficult to establish.

In conclusion, we report HLA class I hyperexpression in thyroid tissue from a large GD cohort consisting both of recent-onset and chronic GD. Additionally, we found the immune response protein STAT1 colocalized with HLA class I and VP1 with PKR, which is indicative of an active, antiviral host response. These results are similar to our recently reported findings in Hashimoto thyroiditis, demonstrating common features in AITD. Moreover, we confirm the presence of VP1 both in chronic and new GD, with VP1 being especially evident in chronic GD. Taken together with earlier reports of MxA, PDCs, and CD8$^+$ T cells in the same tissue samples, we believe our study adds evidence to the theory of a link between enterovirus and thyroid autoimmunity.

Strengths and Limitations

The thyroid tissue cohort used in this study is unique because of its considerable proportion of newly diagnosed GD patients. Moreover, the cohort is large when taking into consideration the limited access to thyroid tissue, especially at the onset of disease. However, this study has several limitations. The size of the control group was limited. We did not analyze PKR and STAT1 in all samples. Moreover, the tissue samples from the chronic GD patients were larger in size because they were taken from surgical specimens.

However, when counting the VP1$^+$ cells, a fraction based on the total number of cells in the tissue sample was used and the proportion of positively stained thyrocytes was used in the HLA class I expression score.

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Author Contributions: T.W. contributed to IHC examinations, data collection, analysis, and interpretation; and drafting of the manuscript. S.S.H. contributed to all parts of the study; study design, clinical coordination and patient recruitment; data collection, analysis, and interpretation; and drafting of the manuscript. T.P. contributed to the surgery and writing of the manuscript. S.J.R. and N.G.M., contributed to the IHC analysis, data analysis and interpretation, and writing the manuscript. K.D.-J., as the principal investigator of the study, had the initial study idea and contributed to the study design; funding; regulatory issues; international collaboration; data collection, analysis, and interpretation; and writing of the manuscript. T.W., S.S.H., and K.D.-J. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Additional Information

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Data Availability: Restrictions apply to some or all the availability of data generated or analyzed during this study to preserve patient confidentiality. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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3

High Prevalence of Common Viruses in Thyroid Tissue

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Abstract

Introduction

Evidence points to viral infections as possible autoimmune thyroid disease (AITD) triggers, but little is known about the prevalence of common viruses in the thyroid gland. Using a novel approach based on virus growth in multiple cell lines followed by detection of the viral genome and visualization of viral proteins, we investigated the presence of multiple human viruses in the thyroid tissue of patients with AITD and in controls.

Methods

Thyroid tissue was collected by core needle biopsy or thyroid surgery from 35 patients with AITD (20 Graves' disease and 15 Hashimoto's thyroiditis). Eighteen thyroid tissue specimens from patients undergoing neck surgery for other reasons than thyroid autoimmunity served as controls. Enterovirus and human herpesvirus 6 were enriched in cell culture before detection with PCR and immunofluorescence, while the remaining viruses were detected with PCR alone.

Results

Forty of 53 cases (75.5%) carried an infectious virus. Notably, 43.4% of all cases had a single virus, whereas 32.1% were coinfected by two or more virus types. Enterovirus was found in 24/53 cases (45.3%), human herpesvirus 6 in 17/53 cases (32.1%) and parvovirus B19 in 12/53 cases (22.6%). Epstein-Barr virus and cytomegalovirus were found in only a few cases. Of five gastroenteric virus groups examined, only one was detected in a single specimen. Virus distribution was not statistically different between AITD cases and controls.

Conclusion

Common viruses are highly prevalent in the thyroid gland. This is the first study in which multiple viral agents have been explored in human thyroid. It remains to be established whether the detected viruses represent causal agents, possible cofactors or simple bystanders.

Introduction

Autoimmune thyroid diseases (AITD), mainly comprising Hashimoto's thyroiditis (HT) and Graves' disease (GD), are the most common autoimmune endocrine disorders and affect 2-6% of the population (1). Predisposing genetic factors include HLA genes and other immunoregulatory genes (2-6). However, environmental factors are believed to play an important part as well. One such environmental factor might be viral infections (7, 8).

Enteroviruses (9-11), parvovirus B19 (12-14) and hepatitis C virus (HCV) (15, 16) have all been associated with AITD. Among herpesviruses, Epstein-Barr virus (EBV) is associated with GD (17-19), while human herpes virus 6 (HHV-6) and cytomegalovirus (CMV) are associated with both HT and GD (20-24) . These aforementioned viruses may produce subclinical disease and persistent infection at any age (25-29).

Case reports and autopsy studies show associations with AITD and additional viruses: rubella virus in congenital illness (30), human T cell leukemia virus (31), Hantaan virus (32), hepatitis E virus (33), HIV (34) and – more recently – Severe Acute Respiratory Syndrome Coronavirus 2 (35-39). In addition, various gastroenteric viruses are more frequently detected in children with autoimmune diseases such as celiac disease and type 1 diabetes (40, 41).

We set out to investigate the presence of widespread viruses in a well-characterized and large collection of thyroid tissue samples. Following indications in literature, we searched for the following agents: enterovirus, parvovirus B19, HHV-6, EBV, CMV, HCV and five gastroenteric viruses (adenovirus, astrovirus, norovirus, rotavirus and sapovirus). The need for an enhanced spectrum of viral detection methodologies and of techniques that could reveal a broad range of viral RNA and DNA genomes in small tissue samples proposed a challenge (42, 43). Results indicate that several common viruses are frequently present in thyroid tissue.

Methods

Study participants, collection of thyroid tissue and blood donors

In this study, we used a previously described collection of thyroid tissue samples at Oslo University Hospital (11, 44). Fifteen HT samples were collected with core needle biopsy, while 20 GD samples were collected from both surgical samples and by core needle biopsy. Thyroid tissue samples from 18 patients undergoing neck surgery for other reasons than AITD, i.e. thyroid tumors or parathyroid adenomas, served as controls. Unrecognized or pre-existing thyroid autoimmunity in controls was excluded by measuring anti-thyroid antibodies. Specimens were snap frozen in liquid nitrogen and stored at - 80°C. The Regional Ethics Committee approved the study (REK no. 1.2006.1950) and written informed consent was obtained from all participants. A group of blood donors (Varese, Italy) was used as a control for evaluating the frequency of the investigated viruses in blood of healthy subjects. The study was approved by the Ethics Committee of Ospedale di Circolo and Fondazione

Macchi (Varese, Italy; 2018/02357094) and performed in accordance with the Declaration of Helsinki and local regulatory laws.

Cell lines and virus entry factors

Five cell lines (AV3, RD, Ht-29, VC3 and HEK293) obtained from The European Collection of Authenticated Cell Cultures and The American Type Culture Collection, were cultured at 37°C in air with 5% CO2 using DMEM/F12 medium supplemented with L-glutamine, 7% heat-inactivated fetal bovine serum and penicillin/streptomycin. Cell cultures were checked monthly for mycoplasma contamination (MycoAlert ™PLUS Mycoplasma Detection Kit, Euroclone-Lonza, Pero, Italy). Expression of enterovirus and HHV-6 cell entry factors was evaluated by indirect immunofluorescence (IF) in uninfected monolayers of each cell line using primary and secondary antibodies listed in Table S1. By indirect IF, the five cell lines above were shown to express the major EV receptors (45, 46), as well as the main two entry factors for HHV-6 (47, 48). For details, see Supplementary Data.

Detection of enterovirus and HHV-6 in thyroid tissue following enrichment in cell culture

The reported mixture of human cell lines that express enterovirus and HHV-6 receptors were used for enriching enterovirus and HHV-6 from the collected thyroid specimens (43, 49). Briefly, thyroid tissue homogenates were produced in medium (containing the PANTA antibiotic mixture) using a FastPrep-24™ blender with glass beads (MP Biomedicals, Eschewege, Germany). Homogenates were cultured in T25 flasks previously seeded with a mix of five cell lines in order to support virus growth. After 2-3 serial passages, DNA and RNA were extracted from each T25 flask (supernatant plus cells) using an automated m2000sp instrument (Abbott Molecular, Rome, Italy). RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase and VILO™ Master Mix (Thermo Fisher Scientific-Invitrogen, Monza,

Italy). End-point PCR assays for enterovirus and HHV-6 were run on Veriti™ Dx thermal cyclers (Applied Biosystems, Thermo Fischer Scientific, Monza, Italy) in a final volume of 50 μl using 15 μl of template (43). Five different sets of enterovirus-specific primers targeting the 5'untranslated region of enterovirus genomes were used (Table S2). Regarding HHV-6, we used primers targeting a conserved sequence comprising the cytoplasmic envelopment protein 2 - tripartite terminase subunit 3 (Table S2). For details, see Supplementary Data. PCR tests were deemed positive when an amplicon of the expected size was observed in the electropherogram. Its viral nature was confirmed by Sanger sequencing. Public databases were employed for attributing the sequences to the appropriate virus species. A schematic presentation of the method is provided (Figure 1).

Detection of enterovirus and HHV-6 antigens in infected cell cultures

Cell cultures in Millicell®EZ slide 4-well glass (Merck-Millipore, Vimodrone, Italy) were incubated with supernatant of T25 flasks that had been exposed to thyroid tissue specimens. After 4-6 days, cell monolayers were fixed in PBS containing 4% paraformaldehyde. Expression of enterovirus protein antigens was evaluated by IF with anti-enterovirus monoclonal antibodies (mAbs) (Table S1) targeting either the VP1 capsid protein (mAbs 9D5, 6-E9/2, 5-D8/1) or the viral 3D RNA polymerase (mAbs 3D-02 and 3D-05; our own laboratory) (50). HHV-6 antigens were detected with a mouse mAb directed at the 140 kDA capsid polypeptide. Enterovirus VP1 staining was deemed positive when fine granular cytoplasmic fluorescence was detected. In persistently infected cells, staining for the 3D RNA polymerase typically produced dotted fluorescence in the nuclear area. The HHV-6 capsid protein antibody produced granular cytoplasmic fluorescence, often perinuclear (Figure 2). For details, see Supplementary Data.

Whole genome amplification and real-time qPCR for viral agents other than enterovirus and HHV-6

The small quantities of thyroid tissue from biopsies posed a challenge in the search for multiple viral agents. Thus, we had to resort to a whole genome amplification (WGA) method for obtaining amounts of DNA templates sufficient for numerous PCR tests. The WGA2 method (Sigma-Aldrich, Milano Italy) was used according to the manufacturer's protocol. While DNA extracted from thyroid tissue was used directly in the WGA2 procedure, before WGA2 the RNA had to be converted into DNA (using SuperScript™ III Reverse Transcriptase and VILO™ Master Mix described on page 6). PCR assays were run in duplicate using 10 μl of WGA2-derived DNA template. A total volume of 25 μl was used for each real time qPCR reaction run on ABI Prism 7500 thermal cyclers (Thermo Fischer Scientific-Applied Biosystems, Monza, Italy). Table S3 shows the qPCR kits that have been used to detect CMV, EBV, parvovirus B19, HCV, adenoviruses F40-F41, astrovirus species 1-8, norovirus genogroups I and II, rotaviruses and sapoviruses. The analytical sensitivity of qPCR tests was 10-100 genome equivalents per reaction.

Peripheral blood leukocytes of blood donors

Nineteen healthy blood donors were used as controls for attempts to detect the investigated viral agents in leukocytes of peripheral blood (a possible virus reservoir in persisting and latent infection). For details, see Supplementary Data.

Results

We investigated the presence of enterovirus, parvovirus B19, HHV-6, EBV, CMV, HCV in addition to five gastroenteric viruses (adenovirus, astrovirus, norovirus, rotavirus and sapovirus). Patient characteristics are summarized in Table 1, and virus detection results are summarized in Table 2. As shown in Table 2, enterovirus and HHV-6 were sought for using pre-enrichment of virus in cell culture before PCR and IF assays. As a control, the investigated viruses were searched for in peripheral blood leukocytes of 19 healthy blood donors and were rarely detected. Enterovirus, HHV-6 and EBV were found separately in three donors; no coinfections were found.

Using PCR alone, enterovirus was detected in 18/53 (34.0%) specimens, but IF for the capsid VP1 protein proved more sensitive with 24/53 specimens (45.3%) resulting positive. Concordant PCR and IF results of enterovirus detection were obtained in 16/53 (30.2%) cases. Enterovirus was more frequent, albeit not significantly so, in the HT group (60.0%) compared to the GD group (50.0%) and the controls (38.9%). Regarding HHV-6, the PCR and IF methods were concordant in 17/53 positive cases (32.1%). The HHV-6 distribution did not differ significantly among the three investigated groups. Figure 2 shows the expression of enterovirus and HHV-6 capsid protein in cell cultures of selected virus-positive genome cases (and confirms that the detected genomes are expressed within thyrocytes). Parvovirus B19 – a prevalent virus with various clinical manifestations (51), amongst them thyroid disorders (7, 12), was only investigated using PCR amplification. Twelve out of 53 cases (22.6%) were parvovirus B19 positive. The detection rate between the groups did not differ significantly.

Among the five taxonomic groups of gastroenteric viruses studied, a single GD case carried modest amounts of a rotavirus strain, indicating that gastroenteric viruses rarely infect the

thyroid gland. Other agents detected in only a few cases include EBV and CMV, two members of the herpesvirus family that are known to cause life-long latent infections in a high percentage of the population. Though the association of HCV with AITD has been demonstrated (15, 16), we did not detect HCV in any thyroid tissue samples.

Surprisingly, however, the thyroid was rarely devoid of virus. As shown in Table 3, 75.5% of the investigated cases carried at least one virus in the gland, suggesting that unapparent viral infection of the thyroid is commonplace. Yet, etiologic/pathogenic studies are initiated with the aim of discovering the possible triggers of disorders that lack an established causal factor. We thus elaborated on coinfections as possible risk factors. Table 4 shows the rate of concurrent virus detection (or coinfection) by enterovirus, HHV-6 and parvovirus B19, the agents found most frequently in our study. A single virus was found in 23/53 cases (43.4%), while 17/53 cases (32.1%) harbored two or three different agents.

The viral nature of PCR amplicons was ascertained by Sanger sequencing. Table 5 shows representative sequences of the enterovirus and HHV-6 strains that were cultured in vitro. Enterovirus sequences (relative to the partially conserved 5'UTR or the VP4-VP2 genome regions) matched those of members of the A and B enteroviral species or - in a few cases – of the rhinovirus C species that is part of the EV genus (52). HHV-6 sequences refer to a strongly conserved genomic tract (CEP2-TRM3). In fact, no differences were perceived among different cases of the control, GD, HT or blood donor groups. Unfortunately, the sensitive PCR method and the antibodies used for HHV-6 detection did not allow discerning the A from the B species of the virus.

Discussion

In this study, we investigated 53 thyroid tissue samples for the presence of different virus species. As far as we know, few, if any, have searched for this wide range of viruses simultaneously in thyroid tissue. We herein show that common viruses are frequently found in thyroid tissue.

Proof of live enterovirus and HHV-6 in thyroid tissue were obtained by genome detection and sequencing in addition to the detection of viral proteins in cultured cells. This study is the first study to assess the presence of enterovirus in thyroid tissue with this method. We found enterovirus in a high proportion of the samples, with a slightly higher detection rate, albeit not significantly so, in both GD and HT compared to controls. There was a slight discrepancy between the two methods applied, however the majority of enterovirus positive samples (61.5%) were positive with both IF and PCR. We previously published that IF may give superior results compared to PCR in the diagnosis of persistent infections such as type 1 diabetes, post-polio syndrome and viral myocardiopathy (43). Similar indications emerge from immunohistochemical studies (53). The huge variation of enterovirus genomes amongst over 200 virus types makes matching primer pairs with genome sequences challenging. The antigenic variation in the enteroviral VP1 capsid protein, which is used in immunohistochemical studies, is however, much lower, making antibody-based enterovirus detection more robust.

Partial sequences of the enterovirus strains found in thyroid hint to the A species (mainly coxsackievirus A) and B species (apparently echovirus types). However, the methods employed cannot correctly identify the infecting virus type since this requires deciphering the VP1-VP2 enteroviral capsid sequences, an aim that could not be reached in this work due

to the minimal viral load present in thyroid even after virus enrichment in cell culture (36, 43). However, surveillance studies and serology show that several enterovirus types of the A and B species are circulating worldwide, often causing subclinical infection (54). More surprising is the finding of members of the rhinovirus C types within the thyroid gland. Currently, rhinoviruses are included in the enterovirus genus (52, 55), and cause the common cold as well as lower respiratory infections (56). A low-grade persistent (not latent as in the case of herpesviruses) enterovirus infection has been shown to be linked to type 1 diabetes and other autoimmune diseases (57-63). Experiments from our group showed that infection of cultured cells with enterovirus strains derived from type 1 diabetes pancreatic samples enhance the expression of pro-inflammatory cytokines and chemokines characteristic of autoimmune disorders (64). In human thyroid, viral infection has also been shown to activate interferon signaling and the expression of interferon-stimulated genes (36, 49). Moreover, we previously found additional viral footprints in the same thyroid tissue collection used in this study (9, 10).

Human herpes virus 6 is divided into two different species: HHV-6A and HHV-6B. HHV-6A is more neurovirulent, and associated with neuroinflammatory disorders such as multiple sclerosis (65). HHV-6B causes *exanthema subitum* (the sixth disease in infancy) and its replication can be reactivated by immunosuppression (66). With the methods applied in this study (both PCR and IF) we could not differentiate HHV-6A from HHV-6B. Thus, we may only conclude that HHV-6 is frequently present in thyroid without identifying the A or B species. Further research is needed in this regard since previous studies suggest that HHV-6 of the A species is likely associated with HT and possibly with other autoimmune diseases (8, 21-23). We could not establish whether the HHV-6 genome was present in a “latent, inert state” in thyroid cells, or being expressed at a slow pace.

Among coinfections detected in thyroid, the association of enterovirus with HHV-6 was the most frequent, followed by HHV-6 plus parvovirus B19, enterovirus plus parvovirus B19 and, sporadically, the association of one of the three main viruses with EBV or CMV (Table 4). It remains an open question whether carrying two or more viruses in the thyroid gland may confer an increasing risk for AITD. It is of interest to recall that HHV-6 is capable of transactivating EBV (67) and that multiple sclerosis risk increases by far when genetically predisposed subjects become doubly infected with HHV-6A and EBV (68). It needs to be established whether dynamics of this type are also operative in AITD.

Even though our results show that the prevalence of viral infections is not statistically different between AITD and controls, we do not believe that this upends the hypothesis that viruses may represent environmental triggers of thyroid autoimmunity. Actually, the findings may add evidence to this possibility proving that multiple viral agents are common within the thyroid gland. Moreover, there is firm evidence of a genetic contribution and genetic vulnerability is probably a prerequisite for developing AITD. In patients genetically predisposed for AITD, an additional AITD susceptibility gene was expressed when exposed to products of microbial infection (69). This interplay of genetic predisposition and infectious insults could explain why common infections give rise to autoimmunity in some but are negligible in others.

We recognize that criteria involving viral nucleic acid or proteins in diseased tissue do not distinguish whether a virus is a causal agent, a causal cofactor or a simple bystander that homes to diseased tissue but does not contribute to pathology. Usually, however, the evidence becomes more significant when different investigators come to the same conclusions. To this end, a search of viruses in thyroid biopsy samples could be both safe and

productive. Studies of this type may provide valuable clues to the prevention and cure of AITD.

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Author Contributions

T.W., A.G., F.B., A.T. performed experiments, analyzed data, and drafted the manuscript. T.H.P. contributed to thyroid surgery and core needle thyroid sampling, and writing of the manuscript. S.S.H. contributed to all parts of the study; study design, clinical coordination and patient recruitment; data collection, analysis, and interpretation; and drafting of the manuscript. K.D.-J, as the principal investigator of the study, had the initial idea of the study and contributed to the study design; regulatory issues; international collaboration; data collection, analysis, and interpretation; and writing of the manuscript. K.D.-J and A.T. obtained funding.

T.W., S.S.H. and A.T. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Furthermore, this study has been performed in full adherence to ethical and legal requirements in Norway and Italy.

Author disclosure statement

The authors have nothing to disclose, and no competing financial interests exist. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Table 1. Patient characteristics

	Control group (n=18)	Graves' disease (n=20)	*P value*	Hashimoto's thyroiditis (n=15)	*P value*
Duration *(months)*	-	12.0 (0.6-24.0)	-	0.0 (0.0-6.0)	-
Female *n(%)*	15 (83.3)	19 (95.0)	*0.247*	15 (100)	*0.102*
Age *(years)*	53.2±13.7	47.5±14.0	*0.209*	45.0±11.1	*0.072*
TSH *(mIU/L)*	1.6 (0.6-2.4)	<0.03 (<0.03-<0.03)	*<0.001*	3.3 (1.4-6.7)	*<0.01*
FT4 *(pmol/L)*	13.3±2.5	27.6±12.8	*<0.001*	12.8±2.7	*0.589*
TRAb *(IU/l)*	<0.09	10.3 (3.6-18.8)	*<0.001*	<0.09	-
TPO-Ab *(kIU/L)*	<35	218.0 (61.0-408.0)	*<0.001*	927.0 (276.0-1500.0)	*<0.001*

FT4, free T4;TPO-Ab, thyroid peroxidase antibody; TRAb, thyrotropin receptor antibody. Reference ranges: TSH 0.5-3.6 mIU/l, FT4 8-20 pmol/l, TRAb <1,5 IU/l, TPO-Ab <35 IU/l.

P values compared to controls, and tested with Student's T-test, Mann-Whitney U test or Pearson's chi-square test.

Duration is the time from diagnosis to biopsy.

Table2. Virus detection results for all 53 cases

		Enteroviruses		Herpesviruses				PV-B19	HCV	Gastroenteric viruses				
Case no.	Group	PCR	IF	HHV-6 PCR	HHV6 - IF	EBV PCR	CMV PCR	PV-B19 PCR	HCV PCR	Adenovirus PCR	Astrovirus PCR	Norovirus PCR	Rotavirus PCR	Sapovirus PCR
1	GD	neg	neg	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
2	GD	**POS**	**POS**	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
3	GD	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg	neg	neg
4	GD	neg	neg	**POS**	**POS**	neg	**POS**	neg	neg	neg	neg	neg	neg	neg
5	GD	**POS**	**POS**	**POS**	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg
6	GD	neg	**POS**	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
7	GD	neg	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
8	GD	**POS**	**POS**	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	**POS**	neg
9	GD	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
10	GD	neg	neg	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
11	GD	neg	neg	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
12	GD	neg	**POS**	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
13	GD	**POS**	**POS**	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
14	GD	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
15	GD	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
16	GD	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
17	GD	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
18	GD	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
19	GD	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
20	GD	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
21	HT	neg	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
22	HT	**POS**	**POS**	**POS**	**POS**	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
23	HT	neg	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
24	HT	**POS**	**POS**	**POS**	**POS**	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
25	HT	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
26	HT	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
27	HT	neg	neg	neg	neg	**POS**	neg	**POS**	neg	neg	neg	neg	neg	neg
28	HT	neg	neg	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
29	HT	**POS**	**POS**	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
30	HT	neg	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
31	HT	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
32	HT	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
33	HT	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
34	HT	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
35	HT	neg	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
36	Ctrl	**POS**	**POS**	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
37	Ctrl	neg	neg	**POS**	**POS**	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
38	Ctrl	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
39	Ctrl	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
40	Ctrl	neg	neg	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
41	Ctrl	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
42	Ctrl	neg	neg	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
43	Ctrl	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
44	Ctrl	neg	neg	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
45	Ctrl	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
46	Ctrl	neg	neg	**POS**	**POS**	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
47	Ctrl	neg	**POS**	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
48	Ctrl	neg	neg	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg
49	Ctrl	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
50	Ctrl	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
51	Ctrl	neg	neg	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
52	Ctrl	**POS**	**POS**	neg	neg	neg	neg	**POS**	neg	neg	neg	neg	neg	neg
53	Ctrl	**POS**	**POS**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Positive samples (n)		18	24	17	17	3	1	12	0	0	0	0	1	0
Positive samples (%)		**34.0%**	**45.3%**	**32.1%**	**32.1%**	**5.7%**	**1.9%**	**22.6%**	**0.0%**	**0.0%**	**0.0%**	**0.0%**	**1.9%**	**0.0%**

HHV-6 , human herpes virus 6; PV-B19 , parvovirus B19; EBV, Epstein-Barr virus; CMV, cytomegalovirus; HCV, hepatitis C virus.

Table 3. Samples with single or multiple viruses detected

	No virus	One virus	Two or more viruses	At least one virus
All samples (n=53)	13 (24.5%)	23 (43.4%)	17 (32.1%)	40 (75.5%)
Controls (n=18)	4 (22.2%)	8 (44.4%)	6 (33.3%)	14 (77.8%)
Graves' disease (n=20)	5 (25.0%)	8 (40.0%)	7 (35.0%)	15 (75.0%)
Hashimoto's thyroiditis (n=15)	4 (26.7%)	7 (46.7%)	4 (26.7%)	11 (73.3%)

Table 4. Concurrent virus detection

	EV + HHV-6	EV+PV-B19	HHV-6+PV-B19
All samples (n=53)	8 (15.1%)	6 (11.3%)	4 (7.5%)
Controls (n=18)	2 (11.1%)	1 (5.6%)	2 (11.1%)
Graves' disease (n=20)	3 (15.0%)	2 (15.0%)	0 (0.0%)
Hashimoto's thyroiditis (n=15)	3 (20.0%)	2 (13.3%)	2 (13.3%)

EV, enterovirus; HHV-6 , human herpes virus 6; PV-B19 , parvovirus B19.

Table 5. RNA or DNA genome sequences representative of the enterovirus and HHV-6 strains obtained from the thyroid tissue of investigated cases.

Case no.	Virus genus	Partial sequence	Genome region	Best-matching virus sequence [1]	Identities	Gaps
43 (CTRL)	Enterovirus	CTCAATCCAGGGGGTGGTGTGTCGTAATGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTCTTATATTGGCTGCTTATGGTGAATTGCAATTACTGTTACCATATAGCTATTGGATTGGCCATCCAGTGACAAACAGAGCTTTGATATACTTGTTCGTGGGTTTCGTTCCACTCA	5'UTR	Coxsackievirus A6 (EV of the A species)	191/198 (96%)	3/198 (1%)
52 (CTRL)		TCCTAAGTTGCAAGCAGATGCCCTCAATCCAGGGGGTGGTGTGTCGTAACGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTCTTACATTGGCTGATTATGGTGACAATTGAGAGATTGTTACCATATAGCTATTGGATTGGCCATCCAGTGATTCAACAGAGCATTGATATACCTGTTTGCGGGTTTTATTCCCACTCTCATCGTTCAGTCCATACTCTAAAGTACATTTTGATTCTGAACAATAGAAAATGGGCGCC	5'UTR	Coxsackievirus A6 (EV of the A species)	260/269 (97%)	2/269 (0%)
53 (CTRL)		CCACTTCAGGGGCCGGAAGAGTGACTAATCCGCATTCAGGGGGCCGGAGGGAATGATTTAAGCCGCATTTCAGAGGGCCGGAGAGAAGATTAGCCGCATTCAGGGGCCGGAGGAAG	5'UTR	Enterovirus A71 (EV of the A species)	45/53 (85%)	5/53 (9%)
34 (HT)		TGCGGAGCACAAGCCCTCAATCCAGGGGTTGGTGTGTCGTAATGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTCTTATATTGGACTGCTTATGGTGACAATTGAGAGATTGTTACCATATAGCTATGGATTGGCCATCCAGTGACAAACAGAGCTTTGATATATTTGTTCGTGGGTTTCGTTCTACTCACCAGTCGTACAGTTCATACTTTAAAGTACATTC	5'UTR	Coxsackievirus A6 (EV of the A species)	239/246 (97%)	2/246 (0%)
36 (CTRL)		TAATTGGTAGTCCTCCGGCCCCTGAATGCGGCTAATCCTAACTGCGGAGCAGACACTCACAATCCAGTGAGCAGTCTGTCGTAATGGGCAACTCTGCAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTCTTATACTGGCTGCTTATGGTGACAATCA	5'UTR	Echovirus E19 (EV of the B species)	161/165 (98%)	0/165 (0%)
45 (CTRL)		TGCGGAGCAGACACCCACGAGCCAGTGGGCAGTCTGTCGTAATGGGTAACTCTGCAGCGGAACCGACTACTTTGGGTGT-CCGTGTTTCA	5'UTR	Echovirus E20 (EV of the B species)	83/89 (93%)	5/89 (5%)
18 (GD)		TCCTCCGGCCCCTGAATGCGCTAATCCTTACTGCGGAGCAGATACCCACAAACCAGTGGGCGGTCAGTCGTATCGGGCAACTCGGCGGCCGAACCGACTACTTTGGGTGTCCGTGTTTCCTTTTATTCCAAATATGGCTGCTTATGGTGACAATTGAAAGGTTGTTACCATATAGCTATTGGATTGGCCATCCGGTAACAAGCAGAGCGATTGTTTATCTGTTTGTTGGTTTCGTTCCATTAAATTATAAAGTT	5'UTR	Echovirus E6 (EV of the B species)	248/255 (97%)	1/255 (0%)
29 (HT)		CACAGATATTGCATGGAAGCCACCATGTGGATAGTCGTAACGGGCAACTGTGGGACGGAACCGACTACTTTGGGTGTCCGTGTTCCTTTTATTCCATTTATTATCTCATGGTGACAACGTATACAGATTATATGTTGTTCACCATGGGCGCTCAGGTATCAAAACAGAATACTGGCTCACATGAAAACGCAATCAATGCTAATAATGGAGGAGTGATCAAATATTTCAACATAAATTATTATAAGGACTCGG	5'UTR	Rhinovirus C (EV of the Rhinovirus C species)	249/253 (98%)	1/253 (0%)
16 (GD)		TCGTCCGTTCCCACAGTTGCCCGTTACGACTATTCCACATGGTGGCTTCCATGCAATTTTTCTGTGGGGTAGGATTATCCCGCATTCAGGGGCCGGAGGAAG	5'UTR	Rhinovirus C (EV of the Rhinovirus C species)	88/97 (91%)	5/97 (5%)
C19 (leukocytes of healthy blood donor)	HHV-6	GTAATTACAATCGACCATCAAAATATAAAGAGCACAGCACTTTTCGCCAGCTGCTACAATACACACGTAAGTACTATTAATTTAACTTTATTTTCAAAAATAAAAAATTAATACATAAAACAACACACAATTAGTCCATTACCACACGCTTACAA	CEP2-TRM3	HHV-6	154/154 (100%)	0/154 (0%)
CTRL cases: 44,36,46,47 ,37,40		GTAATTACAATCGACCATCAAAATATAAAGAGCACAGCACTTTTCGCCAGCTGCTACAATACACACGTAAGTACTATCAATTCAATTTTATTTTCAGAAACAAAAAATTAATACAGAAAATAACGCACACTTAGTCCATTACCACACGCTTACAA	CEP2-TRM3	HHV-6	154/154 (100%)	0/154 (0%)
GD cases: 1,12,2,4,5		CTAATTACAATCGACCATCAAAATATAAAGAGCACAGCACTTTTCGCCAGCTGCTACAATACACACGTAAGTACTATCAATTCAATTTTATTTTCAGAAACAAAAAATTAATACAGAAAATAACGCACACTTAGTCCATTACCACACGCTTACAA	CEP2-TRM3	HHV-6	153/153 (100%)	0/153 (0%)
HT cases: 28,22,29,24		CTAATTACAATCGACCATCAAAATATAAAGAGCACAGCACTTTTCGCCAGCTGCTACAATACACACGTAAGTACTATCAATTCAATTTTATTTTCAGAAACAAAAAATTAATACAGAAAATAACGCACACTTAGTCCATTACCACACGCTTACAA	CEP2-TRM3	HHV-6	153/153 (100%)	0/153 (0%)

[1]Virus sequences attributed to public databases (see Table S1).

5'UTR, 5' untranslated region; HHV-6, Human Herpesvirus 6; CEP2, cytoplasmic envelopment protein 2; TRM3, tripartite terminase subunit 3; CTRl, controls; HT, Hashimoto's thyroiditis; GD, Graves'disease .

Figure Legends

Fig. 1. Model of the integrated procedure used for deriving virus strains from thyroid tissue.

Thyroid biopsy tissues were made into homogenates, and then cultured with five cell lines with proven enterovirus- and human herpesvirus 6 (HHV-6) entry factors. After 2-3 serial passages, RNA (enterovirus) and DNA (HHV-6) were extracted, before viral genome detection by PCR. For each case, the expression of enterovirus and HHV-6 antigens in cultured cells were evaluated by immunofluorescence with a panel of enterovirus and HHV-6 antibodies. Created with BioRender.com.

Fig. 2. Immunofluorescence of enterovirus and human herpesvirus 6 (HHV-6) capsid protein in cell cultures of selected virus positive cases.

Green immunofluorescence of cultured cell monolayers infected with enterovirus (A) and HHV-6 (B) strains isolated from thyroid tissue samples. The monoclonal antibody 6-E9/2 to the capsid protein VP1 was used for enterovirus (A), and a monoclonal antibody specific for the HHV-6 140kDa capsid polypeptide for HHV-6 (B). Red counterstaining with Evans Blue. Cases 2, 8 and 10 are infected with virus derived from GD thyroid tissue samples, cases 28 and 22 derived from HT thyroid tissue samples. Cases 2, 22 and 8 showed the presence of both enterovirus and HHV-6.

Fig. 1.

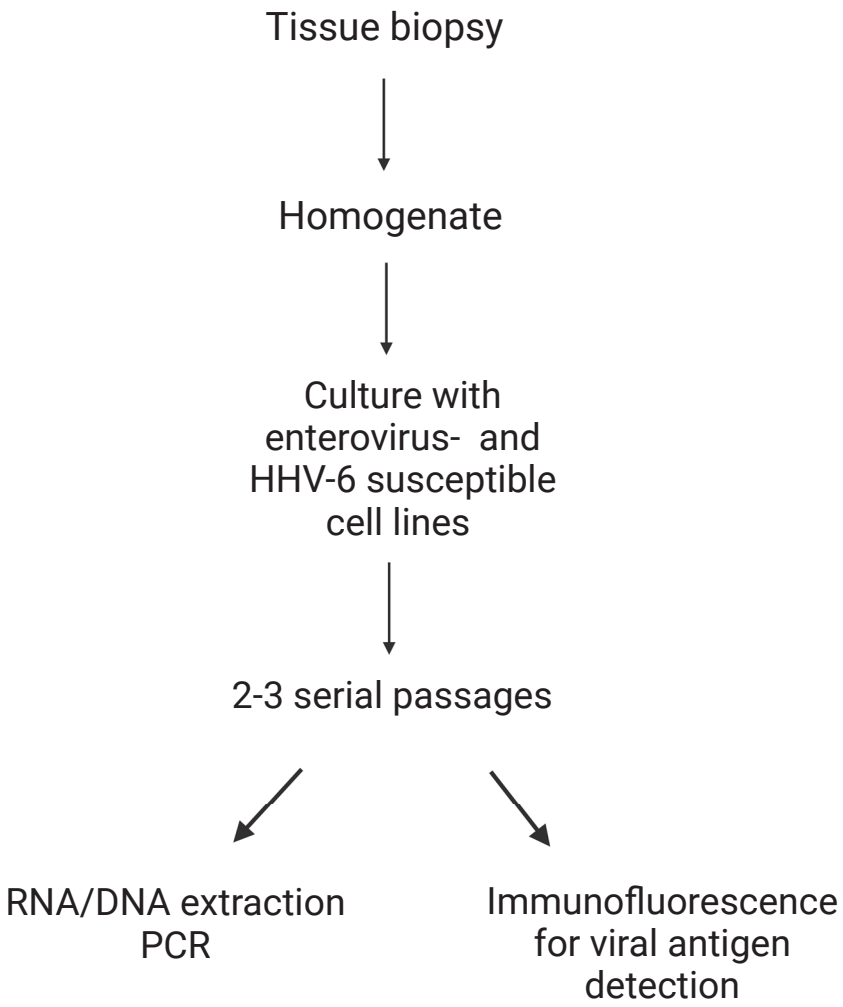
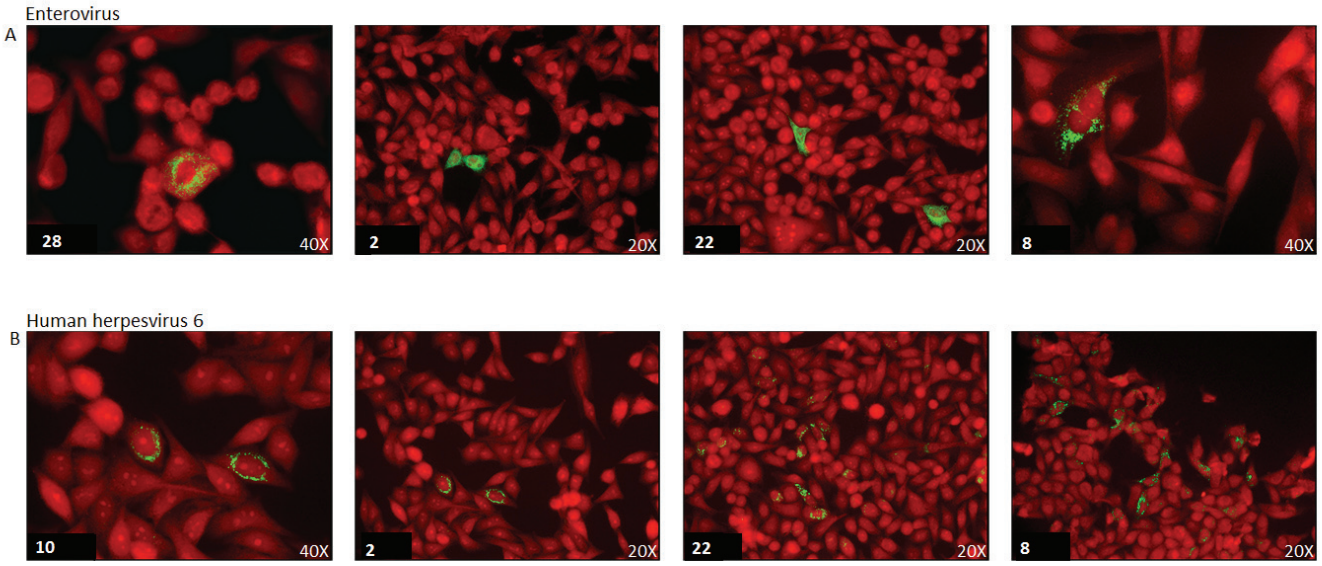

Tissue biopsy
Homogenate
Culture with enterovirus- and HHV-6 susceptible cell lines
2-3 serial passages
RNA/DNA extraction PCR
Immunofluorescence for viral antigen detection

Fig. 2.


A
Enterovirus
28
40X
2
20X
22
20X
8
40X
B
Human herpesvirus 6
10
40X
2
20X
22
20X
8
20X

High Prevalence of Common Viruses in Thyroid Tissue

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SUPPLEMENTAL DATA

Table S1. List of consumables, chemicals, cell lines, viruses, culture media, molecular biology reagents, antibodies, commercial kits, instruments, applications and databases.	
Resource or reagent	**Purchased from**
Plasticware	
Flasks (T-25; T-75); 12-well and 6-well multiplates; cell scrapers; pipettes	Thermo Fisher Scientific-Corning (Monza, Italy)
Millicell EZ 4-well glass slides; Millex®syringe filter units, PVDF pore size 0.22 and 0.10 µm	Merck-Millipore (Vimodrone, Italy)
Cell culture	
Cell lines: AV3, RD, Ht-29, HEK293	European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK)
Cell line: VC3	American Type Culture Collection (ATCC, Manassas, VA)
Fetal bovine serum (FBS); DME/F12 medium with Hepes; L-Glutamine; Pyruvate; Penicillin-Streptomycin; Hank's balanced salt solution (HBSS); Dulbecco Phosphate-buffered saline (DPBS); Trypsin-EDTA	LifeTechnologies-Gibco (Monza, Italy)
Histopaque cell separation medium (density 1.077 and 1.119 g/ml); Bovine serum albumin (BSA); Collagenase type IV; Dispase-I	Sigma-Aldrich (Milano, Italy)
PANTA antibiotic mixture (Polymyxin B; Amphotericin B; Nalidixic Acid; Trimethoprin; Azlocillin)	BD (Milano, Italy)
MycoAlert Plus Mycoplasma Detection Kit; Accutase (cell detachment enzymes)	Euroclone-Lonza (Pero, Italy)
FastPrep-24 - Bead homogenizer for tissue	MP Biomedicals (Eschewege, Germany)
Molecular biology	
GoTaq DNA hot start DNA polymerase and master mix; DNA molecular weight markers; Tris-Acetate EDTA buffer (TAE); Agarose; High-resolution agarose	Promega Italia (Milano, Italy)
Reagents and disposables for the m2000sp automated instrument; DNA and RNA preparation kits; reagent vessels; deep well plates; disposable tips	Abbott Molecular (Rome, Italy)
Custom oligonucleotide primers; Whole Genome Amplification WGA-2 kit; Water (PCR-grade); Tris-EDTA buffer pH 8.0 (TE); low-EDTA (0.1 mM) TE buffer pH 8.0; Elution (10 mM Tris-Cl) buffer EB pH 8.5	Sigma-Aldrich (Milano, Italy)
Superscript III and Superscript IV reverse transcriptase with VILO master mix [containing ribonuclease inhibitor, helper and stabilizer proteins, random hexamer primers OR mixture of random hexamer primers plus oligo (dT)18; dNTPs; $MgCl_2$]; Platinum Taq hot start DNA polymerase and PCR Master Mix; Platinum GC Enhancer	Thermo Fisher Scientific-Invitrogen (Monza, Italy)
Brilliant II SYBR QPCR Master Mix with ROX passive reference dye	Agilent Technologies (Cernusco sul Naviglio, Italy)
GelRed stain	DBA Italia-Biotium (Segrate, Italy)
FlashGel - DNA electrophoresis screening system	Euroclone-Lonza (Pero, Italy)
ABI Prism: end-point Verity Dx thermal cyclers; real-time 7500 thermal cyclers. Gene sequencing: 3500 Genetic Analyzer.	Thermo Fisher Scientific (Monza, Italy)
BigDye Terminator V1.1 Cycle Sequencing Kit; Centri-Spin purification columns	Thermo Fisher Scientific-AppliedBiosystems (Monza, Italy)
LabChip GX Touch 24 Nucleic Acid Analyzer; HT DNA HS Reagents kit dual protocol; Hardshell PCR plate-96 blue	Perkin Elmer Italia (Milano, Italy)
Chemicals (molecular biology grade)	
Ethanol; Isopropanol; Dimethyl sulfoxide (DMSO); N,N-Dimethylformamide; Acetone; Paraformaldehyde 16% ampules (PFA); Triton X100; Tween-20; Na-Azide	Sigma-Aldrich (Milano, Italy)
Antibodies to enteroviruses and herpesvirus type 6; indirect immunofluorescence	
Mouse MAb to the panenterovirus antigen of the VP1 capsid protein, clone 9D5; mouse MAb to HHV-6 A & B envelope glycoprotein gp 60/110, clone	Merck-Millipore (Vimodrone, Italy)
Mouse MAb 5D-8.1 (panenterovirus directed to the VP1 capsid protein)	Dako (Milano, Italy)
Mouse MAb 6-E9/2 Magic (panenterovirus directed to the VP1 capsid protein)	Creative Diagnostics (Shirley, NY)
Mouse MAbs 3D-02 and 3D-05 (panenterovirus directed to the 3Dpol enzyme)	Our own laboratory
Mouse MAb to the HHV-6 capsid polypeptide 140K, clone F24H	Thermo Fisher Scientific (Monza, Italy)
Mouse MAb to the HHV-6 envelope glycoprotein 60/110 kDa	Santa Cruz Biortechnology - DBA Italia (Milano, Italy)
Antibodies to virus receptors (enteroviruses and herpesvirus type 6)	
Alexa Fluor 488 goat anti-mouse IgG; FITC goat anti-rabbit IgG; FITC rabbit anti-goat IgG; ProLong antifade; Evans Blue; DAPI	Thermo Fisher Scientific (Monza, Italy)
Rabbit Mab to CAR (coxsackie virus and adenovirus receptor) clone D3W3G; Rabbit polyclonal to DAF (decay-accelerating factor); Rabbit polyclonal to FCRN (neonatal Fc receptor, echoviruses-specific)	Thermo Fisher Scientific (Monza, Italy)
Rabbit antibody to PVR (CD155); Rabbit antibody to CAR; Rabbit antibody to DAF; Rabbit antibody to Integrin α2β1 (VLA2)	Cell Signaling - DBA Italia (Milano, Italy)
Rabbit antibody to SCARB2; Rabbit antibody to PSGL1; Rabbit antibody to Integrin alpha-V/beta-3 (ITGB3, CD62)	Antibody Genie - DBA Italia (Milano, Italy)
Rabbit antibody to Integrin alpha-V/beta-6 (KREMEN1); Rabbit antibody to CD134 (OX40); Mouse antibody MAb to CD46	Merck-Sigma (Milano, Italy)
Rabbit antibody to Heparan sulfate/Sialic acid; Mouse antibody MAb to ICAM-1 (1A29)	Thermo Fisher-Invitrogen (Monza, Italy)
Mouse MAb to ICAM-1 (CD54)	Novus Biologicals - DBA Italia (Milano, Italy)
Goat antibody to ICAM-5/Telencephalin	R&D Systems - BioTechne (Milano, Italy)
Mouse Mab to CD46 (Membrane cofactor protein) clone MEM-258	Merck-Sigma (Milano, Italy)
Mouse Mab to CD134 (OX-40, member of the TNF receptor superfamily) clone ACT35	Thermo Fisher Scientific (Monza, Italy)
Immunofluorescence microscopy	
Nikon E80i microscope with 10x, 20x, 40x IF objectives	Nikon (Firenze, Italy)
Applications and databases for virus sequences, statistics, graphics	
CLC Main Workbench	Qiagen (Milano, Italy)
https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/	NCBI Virus: viral sequence data from RefSeq, GenBank and other NCBI repositories
http://rvdb.dbi.udel.edu	Reference Viral Database (RVDB)
https://www.viprbrc.org/brc/home.spg?decorator=vipr	Virus Pathogen Resource, J. Craig Venter Institute
viralzone.expasy.org/	SIB Swiss Institute of Bioinformatics
GraphPad Prism 8	GraphPad Software, La Jolla, CA
Photoshop 22	Adobe, San Jose, CA

Table S2. Oligonucleotide primer pairs for detecting enteroviruses (EV) and human herpesvirus-6 (HHV-6).	
Oligo Name	**Sequence 5' to 3'**
EV 5UTR-Tok-F	TCCTCCGGCCCCTGAATGCGGCTAATCC
EV 5UTR-Tok-Rev	GAAACACGGWCACCAAAGTASTCG
EV 5UTR-A-F	GTGTAGATCAGGTCGATGAGTCAC
EV 5UTR-A-Rev	ATTGTCACCATAAGCAGCCA
EV 5UTR-B-F	GACCAAGCACTTCTGTTACCC
EV 5UTR-B-Rev	GTCACCATAAGCAGCCAATATA
EV 5UTR-C-F	GGTGTGAAGAGCCTATTGAGC
EV 5UTR-C-Rev	GATTGTCACCATAAGCAGCCA
EV 5UTR-D-F	TGGTCCAGGCTGCGTT
EV 5UTR-D-Rev	AACACGGACACCCAAAGTAGT
HHV-6-F [1]	GACAATCACATGCCTGGATAATG
HHV-6-Rev [1]	TGTAAGCGTGTGGTAATGGACTAA
1. With reference to sequence of the HHV-6 strain 02-572-M, the HHV-6 primers target nt 102772-102947 comprising partial sequences of the following genes: cytoplasmic envelopment protein 2 (CEP2) - tripartite terminase subunit 3 (TRM3). Amplicon size 175 nt.	

Table S3. Commercial real time PCR kits for detecting multiple viral agents.	
Description	**Purchased from**
Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Hepatitis C virus (HCV)	Abbott Molecular (Rome, Italy)
Parvovirus B19, Astrovirus species 1-8, Norovirus genogroup I and II, Rotaviruses	Altona Diagnostics (Segrate, Italy)
Sapoviruses	Creative Biogene (DBA, Milano, Italy)
Enteric Adenoviruses F40, F41	BD (Milano, Italy)

Cell lines, virus entry factor and reference virus strains.

By indirect immunofluorescence, the AV3, RD, Ht-29, VC3 and HEK293 cell lines were evaluated for the expression of the major enterovirus cell entry factors: CAR; DAF; PVR; Integrin alpha-V/beta-3; Integrin alpha-V/beta-6 (KREMEN1); PSGL1; SCARB2; Heparan sulfate/Sialic acid; ICAM-1; ICAM-5/Telencephalin, as well as the two main HHV-6 receptors CD46 and CD134. The coxsackievirus B3 (CBV3, Nancy strain) and a blood isolate of HHV-6 (C19; Virology Laboratory, Varese, Italy) were used as reference for the detection of enterovirus and HHV-6 genomes, respectively.

Detection of enterovirus and human herpesvirus 6 genomes by gene amplification

Thermocycling parameters were as follows: a) enterovirus (touchdown PCR): (Stage 1 (x 1): 95°C, 5min; Stage 2 (x 10): 95°C 10sec, 64°C (then -1°C per cycle) 10 sec, 72°C 20 sec; Stage 3 (x30): 95°C 10sec, 55°C 10 sec, 72°C 20 sec; Stage 4 (x 1): 72°C, 5 min, 4°C); b) HHV-6 (Stage 1 (x 1): 95°C, 5min; Stage 2 (x 45): 95°C 10sec, 60°C 10 sec, 72°C 20 sec; Stage 3 (x 1): 72°C, 5 min, 4°C). PCR amplicons were identified by molecular size using a LabChip GX Touch 24 analyzer based on capillary electrophoresis (Perkin Elmer, Milano, Italy).

Detection of enterovirus and human herpesvirus 6 antigens in infected cell cultures

For additional virus typing, select monolayers were stained with monoclonal antibodies specific for coxsackieviruses group-B; echoviruses 4, 6, 9, 11, 30, 34; echoviruses 4, 6, 9, 11, 30; polioviruses 1-3 (Table S1). Alexa Fluor 488-goat anti-mouse IgG (Thermo Fisher Scientific, Monza, Italy) was used as secondary antibody. Slides were counterstained with Blue Evans. Images were taken with a Nikon E80i microscope and adjusted in brightness and contrast using Adobe Photoshop (Adobe, San Jose, CA, USA).

Peripheral blood leukocytes of blood donors

Leukocytes (including granulocytes) were obtained from a fresh aliquot of blood by centrifugation on discontinuous Ficoll-Hypaque gradients (density 1.077 and 1.119 g/ml). After washing 2x with medium, the leukocytes were cocultured with the human cell mix and processed as described above for virus detection. A leukocyte aliquot was preserved at -70°C for nucleic acids extraction.

Erratalist e

Navn på kandidat: Therese Weider

Avhandlingstittel: Autoimmune Thyroid Diseases - Traces of Viral Infection

Forkortelser for type rettelser:

Add – addition

Cor – correction

Side	Linje	Originaltekst	Type rettelse	Korrigert tekst
VIII	2		add	Mari Kaarbø and Alexander Bauer Westbye -added to acknowledgment.
XII	26		add	Paper 3 has been published, with minor revisions, in Frontiers in Endocrinology after the thesis was approved.
14	33	...*Enterovirus* genus are separated into 12….	cor	...*Enterovirus* genus is separated into 12….
44	14	...function mutations were first described in 2011v in patients….	cor	..function mutations were first described in 2011 in patients…
45	32	The work of Koch and his collaborator Loeffler, did not, however, write..	cor	Koch and his collaborator Loeffler, did not, however, write...
46	13	...is demonstrated in paper 3 were virus from thyroids…	cor	...is demonstrated in paper 3 where virus from thyroids…
Paper 3. Table 4.		EV+HHV-6 All samples (n=53) – 8(53.3%)	cor	EV+HHV-6 All samples (n=53) – 8(15.1%)