University doctoral thesis (Ph.D.)

Thesis

Associations of autoimmune diseases in autoimmune polyglandular syndromes

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1. Introduction

1.1. APS

Autoimmune Polyglandular Syndrome (APS) is a complex and heterogeneous disease in which endocrine and non-endocrine organ-specific and systemic autoimmune diseases are associated. A diagnosis of APS can be made when at least two organs or organ systems are affected by autoimmunity, at least one of which is endocrine (1,2).

The APS is traditionally divided into four subgroups (3). APS I is a monogenic disorder that develops in childhood as a result of mutations in the autoimmune regulatory gene (AIRE), the concomitant occurrence of at least two of the three diseases Addison's disease (AD), mucocutaneous candidiasis (CMC) and hypoparathyroidism (hypoPT) (3).

APS II is described as consisting of component diseases such as AD, autoimmune thyroid disease (AITD) and/or type I diabetes mellitus (T1D) (3). Patients with AD have at least a 50% lifetime risk of developing additional autoimmune diseases (4). AITDs include Hashimoto's thyroiditis (HT) and Graves' disease (GD) (5).

In APS III, AITD can be associated with any other autoimmune disease except AD (3,6-8).

Patients who do not fall into one of the three categories mentioned above are included in APS IV (3,6-8). According to Gatta et al., T1D is a key element in the diagnosis of APS IV (9). In addition, the literature offers some other classifications that differ in their definition. According to Frommer and Kahaly, there is a juvenile and an adult version of APS that combines groups II-IV of the traditional classification. Most variations have been described for APS groups II and III (1,10).

The APS III group can be further divided into subgroups. Betterle et al. distinguished 4 subtypes based on the associated diseases. The subgroups are 3A): AITD and autoimmune endocrine diseases (T1D, lymphocytic hypophysitis (LH), premature ovarian failure (POF)); 3B): AITD and autoimmune diseases of the gastrointestinal tract, liver and biliary tract or exocrine pancreas (autoimmune gastritis (AIG), celiac disease (CeD), autoimmune hemolytic anemia (AIHA), autoimmune hepatitis (AIH), inflammatory bowel disease (IBD), primary biliary cholangitis (PBC)); 3C): AITD and autoimmune diseases of the skin, nervous system and/or hematopoietic system (vitiligo (Vit), alopecia (Alo), myasthenia gravis (MG), multiple sclerosis (MS)); and 3D): AITD and systemic autoimmune diseases (systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), systemic sclerosis (SSc)) or autoimmune heart disease, antiphospholipid syndrome or vasculitis (11,12). In addition, this working group refers to APS III as multiple autoimmune syndromes (MAS) III (11). The rationale behind this nomenclature is that APS III includes both endocrine and non-endocrine autoimmune manifestations, so that the term "polyendocrine autoimmunity" is inappropriate (11). As is typical for autoimmune diseases, women are more often affected by APS than men. The peak incidence of the syndrome is in the third or fourth decade of life, although the onset of each disease may vary (2,8,13).

APS is characterised by lymphocytic infiltration of affected tissues, the appearance of autoantibodies against endocrine and non-endocrine organs, and disturbances of the humoral and cellular immune response (2,14). HLA, CTLA-4 and PTPN-22 gene involvement has also been observed in APS II and III (15). Both genetic and environmental factors may contribute to the loss of immune tolerance to autoantigens by disrupting communication between antigen-presenting cells and T cells (16). In addition to the endocrine autoimmune abnormalities required for diagnosis, both APS II and APS III may be associated with a number of other non-endocrine autoimmune diseases (8). Autoimmune disorders of the endocrine glands such as AITDs, T1D, AD, POF, hypoPT or LH may be combined with non-

endocrine organ-specific autoimmune disorders including Vit, Alo, IBD, CeD, AIH, AIHA, AIG, PBC, sclerosing cholangitis (SC), MS or MG. In addition, systemic autoimmune diseases such as RA, SLE, SS, psoriasis (PsO), SSc and polymyositis (PM) may be co-morbid (17).

There have been several publications in the literature that have investigated the polymorphisms of HLA and non-HLA genes underlying certain autoimmune diseases in affected populations. If we want to predict autoimmunity in later life, it is essential to elucidate the genetic background. Unfortunately, this was not possible in the present sample, but in the future it will be necessary to perform appropriate genetic tests as a means of clarifying the combinations of diseases.

1.2. Common autoimmune diseases in APS

1.2.1. Hashimoto's thyroiditis

HT, also known as chronic lymphocytic or autoimmune thyroiditis, is the most common organ-specific autoimmune disease affecting the thyroid gland (18). HT is not only the most common autoimmune endocrine disease but also the main cause of hypothyroidism in iodine-deficient areas. The overall prevalence of HT is about 7.5%, 17.5% in women and 6.0% in men, depending on the geographical region. Autoimmune thyroiditis leads to chronic inflammation of thyroid tissue and about 25% of patients may develop hypothyroidism (19).

Typical symptoms include fatigue, intolerance to cold, weight gain and a general decrease in metabolic processes. In children, hypothyroidism may present with goitre, late puberty or growth retardation. HT-induced hypothyroidism becomes more common in older age, with a peak of onset between 40 and 60 years (19).

Since the aetiology of chronic thyroiditis is multifactorial, a possible causal treatment seems to be a complex solution. Thus, the main, widely accepted treatment is L-thyroxine substitution therapy, which is needed when thyroid function is already reduced. The standard substitution protocol with T4 does not reverse the ongoing inflammatory process. There have been some clinical trials evaluating the addition of other agents to standard therapy. One of the agents tested is selenium, which can be interpreted as a crucial cofactor for enzymes involved in the production of thyroid hormones: thioredoxin reductase, glutathione peroxidase and deiodinases type I and II. With immunomodulatory properties, selenium also influences the course of AITD, with some data suggesting that it reduces progression to hypothyroidism. However, the literature is not consistent on this point. Another therapeutic suggestion is vitamin D supplementation, which has been shown to reduce ATPO and ATG antibodies in patients with HT. What is more, a decrease in thyroid stimulating hormone (TSH) levels and an increase in both free T3 (fT3) and free T4 (fT4) levels have been observed (20).

A high percentage of these patients have other comorbidities such as AD, POF, GD, Vit, Alo, pernicious anaemia and T1D. In most cases, the first manifestation of APS may be the T1D-AITD association. In addition, there is a significant increase in the prevalence of other diseases in HT patients: AIG, RA, CeD, SS, MS, SLE (41). The reasons for the association of AITD with other autoimmune diseases are both shared genetic susceptibility and environmental factors.

1.2.2. Graves' disease

GD is the most common cause of hyperthyroidism, accounting for 60-80% of hyperthyroidism cases (21). The overall prevalence of hyperthyroidism is 1.2%, with an incidence of between 20/100 000 and 50/100 000. It is most common in the age group 20 to 50 years. GD is more common in women than in men. GD is caused by TSH receptor-stimulating immunoglobulin (TSI), also known as thyroid-

stimulating antibody (TSAb) (21). In addition to hyperthyroidism, a significant increase in thyroid size is observed in a significant proportion of patients.

Many environmental factors, including pregnancy (especially after childbirth), iodine excess, infections, emotional stress, smoking and interferon alpha may play a role in the development of GD in susceptible individuals.

Most GD patients show the classic signs and symptoms of hyperthyroidism. The onset of the disease depends on the age of onset, severity and duration of hyperthyroidism. In the elderly population, symptoms may be milder or masked and may present with non-specific signs and symptoms such as fatigue, weight loss and new-onset atrial fibrillation. In younger patients, heat intolerance, sweating, fatigue, weight loss, palpitations, diarrhoea and tremors are common. Other features include insomnia, anxiety, nervousness, hyperkinesia, dyspnoea, muscle weakness, itching, polyuria, oligomenorrhoea or amenorrhoea in women and cervical fullness. Palpable goitre is more common in the population under 60 years of age.

Extrathyroidal manifestations of GD include endocrine orbitopathy with signs of eyelid retraction, proptosis, periorbital oedema, chemosis, scleral injection, exposure keratitis. Eye symptoms include eyelid swelling, eye pain, conjunctival redness, double vision. Thyroid dermopathy causes marked thickening of the skin, mainly over the tibiae (pretibial myxoedema), which is rare, occurring in 2-3% of cases. Bone involvement includes swelling of the metacarpals, known as thyroid acropathy. The pathogenesis of the rare manifestations of GD, such as pretibial myxoedema and thyroid acropathy, is poorly understood and is thought to be due to cytokine-mediated stimulation of fibroblasts.

Treatment of GD depends on the presentation of the disease. Treatment consists of rapid control of symptoms and reduction of thyroid hormone secretion. There are three ways to reduce thyroid hormone synthesis. These options are: antithyroid drugs that block the synthesis and release of thyroid hormone, treatment of the thyroid gland with radioactive iodine (RAI), and total or near-total thyroidectomy.

The onset of GD increases the risk of RA and SLE and similarly, if RA or SLE occurs first, the likelihood of developing GD will be higher. The presence of predisposing HLA genes has been identified as the underlying cause. In the literature, co-occurrence of RA and GD is the most common combination (22).

1.2.3. Diabetes mellitus

T1D is an autoimmune disease that leads to the destruction of the pancreatic beta islet cells, creating a metabolic disorder that requires lifelong insulin therapy (23). T1D occurs primarily in genetically predisposed individuals in whom the onset and progression of the disease are triggered by environmental or immunological events.

T1D can develop rapidly and clinically silently over months or years. The former form is seen mainly in childhood, the latter in adulthood. Its progression is determined by the dynamics of autoimmune markers that appear after the onset of autoimmunity.

Viral infections have long been thought to be able to break immunological tolerance to self-antigens. This supports the notion that a viral pathogen can promote the loss of immune tolerance and the development of T1D. Individuals with T1D often have features of a dysregulated microbiome, possibly related to altered gut permeability or viral infection (23).

The typical symptoms of the disease are weight loss with a good appetite, increased thirst and abundant urine, along with fatigue, skin complaints, recurrent infections, especially urinary tract complaints.

The first element of diagnosis is fasting blood glucose measurement. In questionable cases, an oral glucose tolerance test (OGTT) may be performed, although this is not typically necessary in T1D patients, as fasting blood glucose levels are high enough to make the diagnosis. Due to the lack of insulin, patients are usually admitted as an emergency with ketoacidosis.

Treatment is with lifelong insulin analogues, which the patient administers themselves using pens or pumps. Regular self-monitoring is a key step in the therapy.

T1D is often associated with other autoimmune diseases, such as AITD (15%-30%), CeD (4%-9%), AIG (0.3%-5%), AD (0.5%). Less commonly, T1D is associated with RA, AIH, Vit, Alo and PsO. Autoimmune thyroid disease is more common in older age and a positive correlation with duration of diabetes was also found. The presence of autoantibodies against CeD has previously been associated with younger age at diagnosis of diabetes, shorter duration of diabetes and female sex (24).

1.2.4. Addison's disease

Adrenocortical insufficiency (AI) is a condition in which the adrenal glands do not produce glucocorticoids and mineralocorticoids properly due to reduced adrenal function (25). There are 3 main types of adrenal insufficiency: primary (adrenal), secondary (hyphal) and tertiary (hypothalamic).

Primary AI (PAI), also known as Addison's disease, is a rare disease with an incidence of about 100 per million in Europe. PAI is caused by damage to or malfunction of the adrenal glands, resulting in insufficient production of cortisol, aldosterone and DHEA.

The most common cause of primary adrenal insufficiency is autoimmunity; this type is often called autoimmune Addison's disease or simply autoimmune PAI. Autoimmune PAI can present as an isolated manifestation, but in >60% of cases it can also be seen concomitantly with other autoimmune disorders in APS. Autoimmune PAI is associated with an increased risk in association with almost any other autoimmune disease, either related to common genetic risk factors or to treatment with high doses of glucocorticoids commonly used for several autoimmune diseases.

The clinical manifestations of PAI can be grouped according to the hormone deficiency. Symptoms associated with glucocorticoid deficiency include muscle and joint pain, weakness, anaemia, loss of appetite, weight loss and low blood pressure. Maintenance of blood glucose concentration is impaired, causing hypoglycaemia, fatigue and tiredness. Hypercalcaemia is thought to be caused by increased bone resorption due to the elevated TSH often seen in PAI, as glucocorticoids do not exert their inhibitory effect on pituitary TSH secretion (26). Symptoms associated with low aldosterone levels include hyponatraemia, hyperkalaemia, salt craving and abdominal pain. Serum electrolyte disturbances lead to dizziness, nausea, vomiting and low blood pressure. Symptoms such as low energy, reduced sexual responsiveness, lack of libido in women, erectile dysfunction in men are associated with adrenocortical androgen deficiency. Other symptoms of PAI include dry skin and oral mucosa, and areas of increased friction such as palmar creases, the axillary region and patchy hyperpigmentation of the dorsal foot.

Suspicion of adrenocortical insufficiency through clinical signs is the first step to diagnosis. The clinical presentation of patients is often non-specific, which can lead to delayed diagnosis. In routine laboratory investigations, hyponatraemia, hyperkalaemia and hypoglycaemia increase the clinical suspicion of PAI. If PAI is suspected, the next step is to assess adrenocortical function using a diagnostic test: a combined measurement of serum cortisol and plasma ACTH. The most sensitive laboratory marker of adrenal insufficiency is plasma ACTH, which is significantly elevated and associated with low serum cortisol.

The lack of mineralocorticoid production is supported by the elevated plasma renin activity observed with low aldosterone.

The principle of treatment for adrenal insufficiency is to replace the missing hormones. The generally accepted glucocorticoid is hydrocortisone, which is short-acting and converts to cortisol in the body, and the mineralocorticoid is replaced by fludrocortisone. To counteract the rise in morning ATCH in many patients, a long-acting, basal glucocorticoid such as low-dose dexamethasone is also needed. The main goals of treatment are to prevent mortality associated with adrenal crisis, improve the quality of life of patients, achieve optimal glucocorticoid treatment, and avoid co-morbidities caused by glucocorticoid overuse, such as metabolic syndrome.

Autoimmune thyroid disease, both HT and GD, occurs in 50% of patients with primary adrenocortical insufficiency; T1D occurs in 10-15% of such patients. CeD is present in about 5% of patients with primary adrenocortical insufficiency and AIG in 10%. Women with primary adrenocortical insufficiency are at risk of POF, which may occur in their teens (but usually in their twenties or thirties); overall, this disorder characterises about 10% of patients with primary adrenocortical insufficiency (27).

1.2.5. Coeliac disease

CeD is an autoimmune disease that occurs in genetically predisposed individuals who develop an immune reaction to gluten. The disease primarily affects the small intestine; however, clinical symptoms are wide-ranging, with both intestinal and extra-intestinal symptoms (28). The main environmental factor responsible for the development of CeD is gluten. In most countries, the prevalence of celiac disease in the general population ranges from 0.5% to 2%, with an average of around 1%. Diagnosis of celiac disease in adults requires a combination of celiac serology and duodenal biopsy sampling.

The symptoms are characterised by a wide range of different types. Persistent, unexplained abdominal or gastrointestinal complaints, diarrhoea or constipation, slow growth, persistent fatigue, sudden, severe weight loss, unexplained deficiencies of iron, vitamin B12, folic acid and consequent anaemia are the most common symptoms. Investigations towards CeD should be performed in already diagnosed T1D, AITD patients and first-degree relatives of CeD patients.

The mainstay of celiac disease management remains a gluten-free diet. Improvement and resolution of symptoms usually occurs within days or weeks and often precedes normalisation of serological markers and duodenal villus atrophy.

CeD is often associated with other autoimmune diseases, especially in T1D (4-5%) and HT patients, but also occurs in AIH, SS, SLE and SSc patients. Patients with celiac disease are at increased risk of inflammatory IBD; similarly, patients with IBD are at increased risk of celiac disease (29,30).

1.2.6. Autoimmune gastritis

AIG is a chronic inflammatory disease of a progressive nature affecting the mucosa of the oxyntic (acid-producing stomach) section, leading to progressive mucosal atrophy. The destruction of parietal cells leads to an increase in gastric pH and loss of intrinsic factor, which in turn leads to impaired absorption of iron and vitamin B12, causing iron deficiency anaemia and pernicious anaemia, respectively. Indeed, the common clinical presentation of AIG is iron deficiency anaemia, which can occur in up to 25-50% of patients with AIG, while pernicious anaemia can occur in up to 15-25% of patients. Patients with AIG may complain of subtle or non-specific upper gastrointestinal symptoms, particularly dyspepsia, but most patients are usually asymptomatic (31).

The prevalence of AIG is 3-5 times higher in patients with AITD or T1D, while an association between GD and AIG has rarely been reported. In an observational study in Italy, 53% of patients with AIG had any type of autoimmune thyroid disease. In addition, the prevalence of other autoimmune diseases was higher in patients with AIG and concurrent thyroiditis than in patients with AIG alone. Data on the association between AIG and other autoimmune diseases, including hypoPT, RA, MG, CeD, IBD, AIHA, SLE and AIH are sparse and fragmented (31).

1.2.7. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a severe, chronic autoimmune disease that affects 1% of the world's population. HLA-DRB1 is the major player in the genetic association of RA.

It mainly affects the joints, but should be considered as a syndrome that includes extra-articular manifestations such as rheumatoid nodules, lung involvement or vasculitis, as well as systemic co-morbidities (32,33). Disease-modifying antirheumatic drugs (DMARDs) target inflammation and are defined as reducing the progression of structural damage. Non-steroidal anti-inflammatory drugs (NSAIDs), although they reduce pain and stiffness and improve physical function, do not interfere with joint damage and are therefore not disease-modifying. Glucocorticoids have a rapid symptomatic and disease-modifying effect, but are associated with serious long-term side effects.

The available DMARDs can be divided into the following groups: (1) conventional synthetic DMARDs (methotrexate, hydroxychloroquine, and sulfasalazine), (2) targeted synthetic DMARDs (pan-JAK and JAK1/2 inhibitors), and (3) biological DMARDs (tumour necrosis factor (TNF)-α inhibitors, TNF receptor (R) inhibitors, IL-6 inhibitors, IL-6R inhibitors, B-cell-depleting antibodies and inhibitors of co-stimulatory molecules). After diagnosis, methotrexate is first-line therapy, together with folic acid to reduce and avoid side effects (33).

RA, T1D and GD are autoimmune diseases that are frequently encountered and thus may co-occur more frequently than currently known.

A Swedish study observed a significant prevalence of AITD at diagnosis of RA and an increase in the prevalence of AITD in the 5 years prior to RA diagnosis compared to the general population. A common genetic predisposition was suggested by a study that found a high prevalence of HLA-A24, DR3 and DR4 antigens in patients with RA and HT. Anti-thyroid antibodies were found in 11% of RA patients, with a wide variation of between 2% and 32%. In addition, a higher prevalence of RA was reported among same-sex siblings with AITD compared to siblings without AITD (34).

1.2.8. SLE

Systemic lupus erythematosus (SLE or lupus) is a condition in which the immune system attacks healthy cells and tissues throughout the body (35). Immune activation in SLE is characterised by excessive B-cell and T-cell responses and loss of immune tolerance to self antigens. Constitutional symptoms such as weight loss, fatigue and low-grade fever are common and may be accompanied by arthralgias or arthritis. Arthritis in lupus is characterised by prolonged morning stiffness and mild to moderate swelling of the joints. It is non-erosive, may be symmetric or asymmetric and may affect large or small joints. Skin manifestations are common and may occur in up to 75%-80% of patients. Cytopaenia is common in patients with lupus. Renal involvement is also a common target organ manifestation; the prognosis is poor due to the high risk of organ failure. Primary or secondary involvement of the respiratory system may occur in lupus. Symptoms and response to treatment vary depending on the anatomical site involved. Neuropsychiatric SLE manifestations can be caused by vasculopathy, autoantibodies and inflammatory mediators and may include headache, aseptic meningitis, vasculitis, movement disorders,

seizures, cognitive impairment, psychosis, demyelinating disease, myelopathy, autonomic dysfunction and peripheral neuropathy. Ophthalmic manifestations include keratoconjuctivitis sicca, keratitis, episcleritis, scleritis, uveitis, retinal vasculitis, retinal artery or vein occlusion, retinopathy and several other less common manifestations. Digestive symptoms may include loss of appetite, nausea, vomiting, abdominal pain and diarrhoea (35).

Clinicians use a wide range of drugs to treat lupus, including glucocorticoids, antimalarials, non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressive agents, biological agents targeting B cells and type I interferon receptor inhibitor biological therapy (anifrolumab).

SLE often co-exists with other autoimmune diseases: SS, SSc, MG, myostitis, RA, SM, T1D, IBD, AIH, AITD may all be comorbidities of SLE. This polyautoimmunity is common among SLE patients, affecting up to 41% of patients. Unfortunately, very little literature is available on most disease comorbidities (36).

1.2.9. Sjögren's syndrome

Sjögren's syndrome (SS) is a systemic autoimmune disease that causes inflammation of the exocrine glands, mainly the salivary and lacrimal glands, but also the nose, upper airways, pharynx and, in women, the vagina (37). The main consequence of this inflammation is the development of sicca symptoms, such as dryness of the mucosal surfaces, especially in the mouth and eyes.

Diagnostic tests to determine the symptoms of sicca attempt to measure the dysfunction of the glands and quantify the extent of involvement of the main glands affected. The main ophthalmological tests include Schirmer tests and examination of the surface of the cornea with dyes (fluorescein and lysamine green) that stain degenerated or dead cells (corneal staining). Ultrasound and MRI are used mainly to assess the most common complications of SS, such as infections and lymphoma. Autoantibodies are the most important serological markers of autoimmune diseases. ANAs are the most commonly detected autoantibodies in patients with SS (>80% of patients) and as such are one of the best ways to identify SS.

SS-associated polyautoimmunity is typically associated with RA (3-55%), SLE (5-22%), SSc (14-60%), myositis (10-23%), HT (5%) and GD (3%) (38).

1.2.10. Vitiligo

Vitiligo (Vit) is an acquired pigmentation disorder characterised by the loss of melanocytes, resulting in white patches or leucoderma. The characteristic lesion is a completely amelanotic, non-scaly, chalky-white macula with well-defined margins. Vit is the most common depigmenting skin disease, affecting 0.1-4% of the population worldwide. Men and women are equally affected, although women and girls are more likely to seek advice, probably due to the greater negative social impact compared to men and boys (39,40).

Vit is a multifactorial disorder characterised by the loss of functional melanocytes. The mechanism is complex, involving genetic, autoimmune responses, oxidative stress, production of inflammatory mediators and melanocyte detachment mechanisms.

The diagnosis of Vit is usually straightforward, clinically based on the finding of acquired, amelanotic, non-scaly, chalky-white macules with a characteristic distribution and distinct margins: perioral, labial and distal limb apices, penile, segmental and friction areas. Treatment options include light therapy, topical and systemic immunosuppressants and surgical techniques, which are currently in the

experimental stage, but together can help to halt the disease, stabilise depigmented lesions and stimulate repigmentation.

Due to its autoimmune background, Vit is also often associated with other diseases. It is most commonly seen alongside T1D, AITD, Alo, RA, SLE, SS PsO and AIG. Unfortunately, the literature on comorbidities is scarce (41).

Table 1 shows the antibody abnormalities for the most common manifestations, which are the cornerstones of screening and follow-up.

Table 1 The 10 most common diseases and their autoantibodies

Most common pathologies	**Typical antibody**
Hashimoto thyroiditis	thyroid peroxidase (ATPO) and thyroglobulin (ATG) antibodies
Graves' disease	TSH receptor antibody (TRAb)
Diabetes mellitus	islet cell antibody (ICA), glutamic acid decarboxylase (GAD), islet antigen antibody (IA2), zinc transporter 8 antibody (ZnT8A) and insulin antibody (IAA)
Addison's disease	anti-21-hydroxylase and anti-17-a-hydroxylase antibody
Coeliac condition	tissue transglutaminase, deaminated gliadin and antibody against endomysium
Autoimmune gastritis	parietal cell and antibody against intrinsic factor
rheumatoid arthritis	autoantibodies to rheumatoid factor (RF) and anticitrullinated peptide (ACPA)
SLE	Antinuclear antibody (ANA), autoantibodies against double-stranded DNA (dsDNA) or ribonucleoprotein complexes (Ro, La, Smith and RNP)
Sjögren's syndrome	SSA antigen (also known as antiRo antibodies) and SSB (also known as antiLa antibodies)
Vitiligo	No routinely usable antibody

2. Purpose of our work

1) Summary and evaluation of available literature on APS patient data

2) Comparison of APS groups by age and gender based on literature data

3)Detection of a combination of autoimmune diseases in APS II and APS III based on literature data

4) To assess the differences between HT and GD in terms of associated autoimmune diseases in APS II and APS III patients based on literature data

5) Identification of AD-related and unrelated pathologies in APS Group II based on literature data

6) Grouping of patients with multiple endocrine autoimmune pathologies at the tertiary centre into APS groups

7) Analysis of data from these patients in terms of age at onset, gender and associated autoimmunity

8)Networked detection of disease combinations, focusing on the most common and potentially fatal pathologies

9) Reinterpretation of the APS definitions

10) Initiate a financially feasible screening protocol based on the most common and interrelated manifestations

3. Studies

3.1. Prevalence of autoimmune diseases in APS II and APS III based on literature - meta-analysis

3.1.A. Methods

Using the Medline and Embase electronic catalogues, we searched for the keywords "autoimmune polyglandular syndrome", "autoimmune polyendocrinopathies", "autoimmune polyglandular syndrome type II" and "autoimmune polyglandular syndrome type III" without using language filters. EndNote software was used to manage the selected articles. Among the articles found in the databases, we used those that (1) included data from patients with two or more autoimmune diseases and (2) described at least 10 cases at a time. Review and case-control type publications could not be used. We then created an Excel spreadsheet from the following data: year of publication, type of publication, number of patients reported in the article and diagnosed with APS, mean age, sex, main pathologies (AD, AITD, T1D, POF, Vit, Alo, CeD, AIH, AIG, IBD, PsO, SS, RA, MG, SM, hypoPT, LH) and their combination. Data were synthesised using methods recommended by the Cochrane Collaboration working group. Event rates (percentage distribution) were calculated for dichotomous outcomes. For all analyses, we used a random error model with DerSimonian-Laird estimation. Statistical heterogeneity was analysed using I 2 and $\chi 2$ tests to obtain probability values; $p<0.1$ was defined as indicating significant heterogeneity. Statistical analyses were performed using Stata 15 (Stata Corporation, College Station, TX, USA).

3.1.B. Results

A total of 479 articles matching the search were found in the electronic databases. After filtering for duplicates, 454 publications remained, of which 18 studies meeting all criteria were found after selecting case studies. In these 18 articles, a total of 1312 patients' data were included.

A total of 9 publications reported age-specific characteristics, with a mean age at onset of 34.7 years (95% CI: 22.75 - 46.64 years) for patients with APS.
The gender distribution was examined in 12 articles, with a significantly higher prevalence of APS *in* women (75% [95% CI: 68% - 81%] vs. 25% [95% CI = 19% - 32%], $p<0.001$).
A total of 842 cases were distinguished between APS II (n=177) and APS III (n= 665) (21.1% vs 78.9%).

The most common autoimmune diseases were AITD, T1D and AD (970, 697 and 174 cases respectively).

In APS II, AD was combined with AITD in 114 cases, T1D in 32 cases and both in 18 cases. In addition, one endocrine (POF) and 5 non-endocrine organ-specific diseases (AIHA, Alo, CeD, MS, Vit) occurred in this group. In APS III, 2 endocrine (POF and T1D), 6 non-endocrine organ-specific (Vit, AIH, AIHA, MG, CeD, Alo) and 4 systemic autoimmune diseases (RA, SLE, SS, PsO) were described.

We were also curious about the maximum number of combinations that could occur in one patient. On this basis, we divided the patients into 3 groups: those with two, three or more manifestations. The prevalence of two combinations was significantly higher in APS II than in APS III (476 (75.7%) vs 152 (24.3%), $p<0.001$). For three combinations there was no significant difference between the two groups (33 (44.6%) vs 41 (55.4%), $p=0.739$), whereas the co-occurrence of more than three autoimmunity was only seen in the APS II group.

Among AD patients, 174 combinations were diagnosed. In 83.9% of APS II patients, AD occurred in a dual combination. Among these patients, only AITD and T1D occurred in dual combination, 114 and 32 cases, respectively. A triple combination was diagnosed in 18 cases (10.34%), of which only 13

patients (7.5%) developed the classic triad of APS II (AD, AITD and T1D). In addition to these diseases, Alo, POF, CeD, AIHA, MS and Vit developed in addition to AD. Based on our previous studies, we found that some diseases do not occur in combination with AD. Our meta-analysis showed that AD did not combine with IBD, AIH, hypoPT, LH, MG, PsO, RA, SLE and SS.

3.1.C. Conclusions

APS is a rare, complex group of conditions in which several autoimmune diseases occur simultaneously. To our knowledge, this is the first meta-analysis in the field of APS. APS II is usually associated with AD, AITD and T1D. In APS III, AITD is typically combined with non-endocrine autoimmune diseases.

Our results show that 83.9% of APS II patients had AD in dual combinations, while the proportion of triple combinations was 10.34%. The combination of AD, AITD and T1D was found in 7.5% of APS II patients.

Diagnosis is often delayed because some diseases appear decades apart. The majority of APS cases involve two autoimmune diseases, complicating classification and treatment. The disease is more common in women, and APS III was more common than APS II.

There is a lack of comprehensive studies and patient registries that could help monitor comorbidities and improve diagnostic protocols. Patients with multiple autoimmune diseases are often treated by different specialists, resulting in fragmented care. Screening for organ-specific autoantibodies is costly but essential for early detection and treatment.

In conclusion, our meta-analysis suggests that the association of non-criterion diseases, and thus the emergence of non-standard APS groups, is not an uncommon process. AITD, AD and T1D are the most frequently combined diseases, so a rapid and accurate screening protocol focusing on these and their combinations is essential.

3.2.Analysis of other autoimmune diseases associated with Hashimoto's thyroiditis and Graves' disease based on the literature - systematic review and meta-analysis

3.2.A. Methods

The methods are the same as those described in 3.1.

3.2.B. Results

The characteristics of the included studies are the same as those used in the previous meta-analysis.

Patient characteristics

The distinction between APS II and APS III was made in 842 cases, of which 177 were APS II and 665 APS III (21.1% vs 78.9%).

Prevalence of HT, GD and AITD in APS

Of the total APS patients (1312 cases), 739 (87.8%) reported autoimmune thyroid disorders. The second and third most common autoimmune diseases were T1D and AD (523 and 137 cases, respectively). HT and GD were isolated in 279 and 151 cases, respectively. The diagnosis was AITD in 309 cases without further characterization. The prevalence of HT, GD and AITD did not differ significantly among all APS patients (HT: 35%, 95% CI: 16% - 57% vs GD: 10%, 95% CI: 1% - 22% vs AITD: 10%, 95% CI: 0% - 31%, p=0.064).

No significant differences were found in the prevalence of HT, GD and AITD in APS II patients (HT: 10%, 95% CI: 0% - 35% vs GD: 2%, 95% CI: 0% - 22% vs AITD: 39%, 95% CI: 5% - 79%, p=0.183)

Among patients with APS III, the prevalence of HT, GD and AITD was also not significantly different (HT: 45%, 95% CI: 14% - 78% vs GD: 9%, 95% CI: 0% - 27% vs AITD: 30%, 95% CI: 1% - 72%, p=0.121).

Differences in co-occurrence of autoimmune diseases between HT and GD

Three autoimmune endocrinopathies (T1D, AD and POF), five non-endocrine organ-specific diseases (AIH, AIHA, Vit, MS and CeD) and two systemic autoimmune diseases (RA and PsO) were reported in HT patients.

GD patients were diagnosed with three autoimmune endocrinopathies (T1D, AD and POF), five non-endocrine organ-specific (AIH, Vit, CeD, MG and Alo) and three systemic autoimmune diseases (SLE, RA and SS).

Also, three autoimmune endocrinopathies (T1D, AD and POF), four non-endocrine organ-specific autoimmune diseases (AIH, AIHA, Vit and CeD) and no systemic autoimmune diseases were found in AITD patients.

The assessment of associated autoimmune diseases is further complicated by the lack of proper classification of HT and GD. In this part of the analysis, many cases were classified in the AITD group.

The frequency of each autoimmune disease is presented in Table 2. Only T1D and AD occurred in higher proportions among APS patients diagnosed with autoimmune thyroid disease, 70.7% and 18.5% respectively. The prevalence of AIH, AIHA, Vit, POF, CeD and RA ranged from 1-4%, all others were rare, <1%.

One, two and more than two other autoimmune disorders were combined with any autoimmune thyroid disorder in 91.8%, 8% and 0.1% of cases, respectively.

HT, GD and AITD in dual combination occurred in 167, 92 and 419 cases (24.5%, 13.5% and 61.8%). The prevalence of HT, GD and AITD dual combinations did not differ (HT: 35%, 95% CI: 11% - 63% vs GD: 10%, 95% CI: 0% - 26% vs AITD: 29%, 95% CI: 3% - 63%, p=0.186).

Triple combinations were less common in GD and did not differ between HT and AITD (GD: 3%, 95% CI: 0% - 18% vs. AITD: 77%, 95% CI: 21% - 100%, vs. HT: 14%, 95% CI: 0% - 66% p=0.028).

More than two other autoimmune diseases have never been reported with GD and AITD, a clinical situation that only occurred in HT, but the small number of cases did not allow a relevant statistical analysis.
In HT patients, the most common associated disorders were T1D, AIH and AIHA, with 121, 23 and 19 cases, respectively. GD was most commonly associated with T1D, AD and Vit (64, 7 and 6 cases, respectively). In the group of unclassified AITDs, T1D, AD and Vit were the most common combination (282, 104 and 16 cases, respectively).
The pattern of non-endocrine dual combinations of autoimmune diseases differed slightly between GD and HT; systemic autoimmune diseases - SLE, SS, RA - in dual combination occurred only in GD. In HT patients, systemic autoimmune diseases (RA and PsO) were only part of the triple and even multiple combinations.

Of the non-endocrine organ-specific autoimmune diseases, MG and Alo only occurred in double combination in GD, AIG in AITD and Vit in HT. Interestingly, the triple combination of any autoimmune disease occurred only in patients with APS II in GD. In patients with AITD, a triple combination was only observed when T1D was one of the associated diseases.

3.2.C. Conclusions
In this systematic review and meta-analysis, we sought to identify the typical pattern of HT and GD comorbidities to help better inform patients and clinicians and to initiate a screening algorithm.
The best characterised combinations of autoimmune diseases in patients with APS (3). Unfortunately, publications in this area are mainly case reports, with only 18 publications suitable for further analysis, and the number of published APS patients was low. In APS II and III patients, AITDs are the most common autoimmune disease (87.8%). Finally, only 739 patients with AITDs with known co-associations of other autoimmunity were collected. In addition, only 58% of patients were initially classified as HT or GD, and even this classification was confounded when discussing combinations, leaving only 39% of patients available for analysis of autoimmune combinations. This significantly limits the possible conclusions and highlights the urgent need for better documentation.
We found no difference in the prevalence of HT, GD and AITDs between APS patients in either APS II or APS III. Associated endocrine, non-endocrine organ-specific and systemic autoimmune diseases occurred in both HT and GD. Only T1D and AD were common comorbidities, with a prevalence of <4% for other autoimmune diseases. This should be taken into account for the development of cost-effective screening algorithms.

The prevalence of dual combinations was similar in HT, GD and AITD, but triple combinations were less frequent in GD. Three or more autoimmune disorders occurred in combination only in HT. Systemic autoimmune diseases were diagnosed in double combinations with GD and in triple or more combinations with HT. Several systemic autoimmune diseases may be associated with AITD. Organ-specific, non-endocrine autoimmune diseases (such as AIG, CeD, AIH, MS, MG) are also common co-morbidities with AITD. This means that appropriate screening protocols for AITDs may be useful for patients with systemic autoimmunity and organ-specific autoimmune diseases (42).

A previous review found that HT is significantly more common in individuals with other autoimmunity such as AD, T1D, RA, or SLE. In our meta-analysis, T1D, AIH, AIG, POF, AD, Vit, CeD, MS, RA, and PsO were included in HT patients.

A recent study showed that the following autoimmune diseases were observed with significantly higher frequency in GD patients: T1D, CeD, AIG, Vit, RA, SS, SLE, sarcoidosis, hepatitis C virus-associated mixed crioglobulinemia, polymyalgia rheumatica and MS (43,44). In our review, T1D, POF, AD, AIH,

Vit, CeD, MG, Alo, SLE, RA and SS were diagnosed in GD patients. Interestingly, in GD patients, a triple combination occurred only when AD was one of the associated disorders.

Both HT and GD are due to polymorphisms in the HLA DQ/DR regions. Immunoregulatory genes such as the HLA region, CD40, CTLA4, PTPN22 and FCRL3 are involved in the development of GD (44). The identification of polymorphisms in immunoregulatory genes may be useful in estimating the risk of HT, GD and other combined autoimmunity in APS patients.

In conclusion, a systematic review and meta-analysis could not establish a distinct pattern of autoimmune diseases associated with HT and GD in APS patients, partly due to the lack of an appropriate classification of AITDs in the literature. Better documentation of databases and registries may help to address this issue. Many organ-specific endocrine and non-endocrine and systemic autoimmune disorders can occur in patients with autoimmune thyroid disease, but only T1D and AD have been reported to occur with high frequency in APS patients.

3.3 Network mapping and analysis of diseases in autoimmune polyglandular syndromes

3.3.A. Methods

Our study was performed at the Department of Endocrinology and Metabolism of the University of Pécs Clinical Centre, Department of Internal Medicine, I. Sz. The data were obtained from the MedSol database with the following International Statistical Classification of Diseases and Related Health Problems (ICD/BNO) codes since March 2007. Medical records and charts of outpatients were reviewed and patients with at least two confirmed autoimmune diseases were selected. The patients' data were organised in an Excel spreadsheet (Microsoft Excel 2021, v. 2405), in which the following were entered: age at diagnosis of the first manifestation of APS, sex, APS classification, first and second manifestation of APS, and the time elapsed between diagnosis of these two manifestations. Where present, the third and fourth manifestations of APS were also recorded. In addition, all autoimmune diseases present in the cohort are listed in Table 2.

Table 2. 28 autoimmune diseases in our patient population

Endocrine autoimmune diseases	**Non-endocrine, organ-specific autoimmune diseases**	**Systemic autoimmune diseases**
Hashimoto thyroiditis (n= 256)	Coeliac disease (n=72)	Rheumatoid arthritis (n=45)
Graves' disease (n=102)	Autoimmune gastritis (n=40)	Sjögren's syndrome (n=26)
Diabetes mellitus (n=79)	Vitiligo (n=40)	SLE (n=21)
Addison's disease (n=43)	Ulcerative colitis (n=17)	PsOriasis (n=19)
Early ovarian depletion (n=14)	Crohn's disease (n=14)	Polymyositis (n=6)
Hypoparathyroidism (n=3)	Alopecia (n=11)	Systemic sclerosis (n=4)
Lymphocytas hypophysistis (n=2)	Autoimmune hepatitis (n=9)	Primary antiphospholipid syndrome (n=2)
	Myasthenia gravis (n=8)	
	Primary biliary cholangitis (n=8)	
	Multiple sclerosis (n=6)	
	Autoimmune haemolytic anaemia (n=3)	
	Sclerotizalo cholangitis (n=2)	
	Immune thrombocytopenia (n=1)	
	Chronic mucocutaneous candidiasis (n=1)	

All the analyses performed in the study and their code are available on GitHub via the following link: https://github.com/peterkaltenecker/APS_-_A_comprehensive_analysis.

3.3.B. Results

A Pécsi Tudományegyetem Klinikai Központ I. Belgyógyászati Osztály Endokrinológiai és Anyagcsere Osztályán nyilvántartott, összesen 7559 esetet tartalmazó adatbázisból 380 betegből álló mintát elemeztek, amely tartalmazta a 2007 és 2018 között a rendszerben megjelent összes beteget, pajzsmirigy túlműködés, autoimmun thyreoiditis, primer mellékvese elégtelenség, AD, autoimmun polyglandularis szindróma, T1D, POF, LH, hypoPT diagnózissal. Patients were followed up until 31 December 2023. 3180 patients (49.06%) were diagnosed with autoimmune endocrine disorders. 12% of patients had more

than one autoimmune manifestation, at least one of which affected the endocrine system, and were therefore classified as APS.

Our previous data have shown that patients with AD are the most frequently diagnosed patients with APS (58.9% of 73 AD patients also had a second manifestation), followed by T1D, HT and GD patients, with 26.4%, 13.7% and 5.3% respectively.

Hashimoto's thyroiditis was one of the manifestations in 67.4% of people diagnosed with APS, while the two AITDs together occurred in 94% of patients

According to the diagnostic criteria for APS, most autoimmune diseases in the study population were related to the endocrine system, while the gastrointestinal system was the second most frequently affected. Systemic autoimmune diseases such as RA, SS and SLE were the third most common component disease group in our population. Skin conditions were also present in significant numbers.

It is important to note that, due to the characteristics of the study, we worked with a snapshot in which not all possible manifestations were seen in all cases, but at least the presence of the first two manifestations could be identified, which is a necessary condition for the diagnosis of APS. Consequently, in our population, two manifestations are the predominant scenario, while in 17.4% of cases three and in 3.7% of cases four manifestations were found.

The ten most common diseases were HT, GD, T1D, CeD, RA, AD, AIG, Vit, SS and SLE. The most common condition in terms of both primary and secondary manifestations was HT. A similar trend was observed in the prevalence of GD among manifestations, although with a reduced prevalence. T1D was significantly more likely to manifest as an initial symptom of APS than as a superposed or additional condition. Notably, AIG was the most common as a third manifestation, ahead of other commonly observed conditions including HT, GD and T1D. AIG and SS were less likely to be the initial manifestations of APS.

At the onset of APS, two peaks were observed in both sexes around the ages of 20 and 40 years, with a consistent age difference of 7-8 years between men and women

In terms of age, all age groups were represented in the study population at the onset of APS, from the youngest patients aged 1 year to a maximum age of 83 years at disease onset. The mean age of onset of APS was 31.8 years in the total study population, with a median of 32 years. Females had a higher prevalence, accounting for 84.2% of all patients compared to 15.8% for males. The mean age of women was 32.9 years with a median of 33 years, while the mean age of men was 26 years with a median of 25 years. The data show that on average, the disease occurs 7-8 years earlier in men than in women. For both sexes, two peaks were observed around the ages of 20 and 40 years, indicating an increased incidence of the disease, in line with the age difference between men and women mentioned above.

As mentioned earlier, the two manifestations were the most common in our study and are usually identified together when patients consult a doctor for an accumulation of clinical symptoms, so in 67 cases the time between the first and second manifestation was recorded as 0 years. In addition, there is a wide range of scenarios in which the time between the first and second manifestation can vary between 1 and 53 years. If we break down this long time span into 10-year periods, we can see that in the first 10 years, 65.5% of patients have a second manifestation, in the second 10 years 17.6% develop a second manifestation, while this figure is 8.4% in the 21-30 year period. In 4.4% of patients, the second comorbidity appeared 31 years after the first manifestation.

Of the diseases that make up APS, type 1 diabetes mellitus and coeliac disease are the earliest to present when the first symptom is

There are significant differences in the age at which autoimmune diseases first appear. The two most common first manifestations were T1D and CeD, with an age distribution where the majority of cases occurred under the age of 20 years. T1D appeared significantly earlier than other common first manifestations such as HT, GD, AD, RA and SLE. CeD was second and Vit third in this comparison. No statistically significant difference was seen between HT, GD, AD, RA and SLE. When the data were disaggregated by sex, it was clear that the onset of AD as an initial symptom typically occurred at an earlier age in men.

Age at onset of the ten most common disorders as first manifestations of APS, by sex. Network analysis of disease associations in APS

A total of 113 disease combinations were detected, 46 of which occurred more than once, highlighted in Figure 14.A. Combinations that were unique account for 17.6%. Many combinations were very rare, but those that occurred 5 or more times accounted for more than half of the population (63.9%). Narrowing it down further to combinations occurring 10 or more times, the range still represented 51.3%. These most frequent combinations consisted only of dual manifestations of the 10 most frequent diseases listed above. Due to the nature of the data collection, combinations of 3 or more diseases are less represented.

An important, but not surprising, insight from the network mapping is that HT was in no way linked to GD. Due to their biologically opposite nature, the co-occurrence of the two diseases was not detected in any of the patients. The most frequent associations were related to HT, with T1D, CeD, RA, Vit, AD, SLE, AIG or SS in descending order. The second most frequent group of co-occurrences involved GD associated with RA, CeD or T1D. The clustering algorithm effectively distinguished HT and GD in the network and sorted the associated disorders so that they were more densely associated within their own community than with nodes in other communities. Yet the related disorders of HT and GD were not completely separated. HT and GD share many comorbidities, 15 in number. There was only one disease that was associated with GD but not with HT (ITP), 6 that were associated with HT but not with GD (Alo, POF, AIH, PAPS, AIHA), and 3 that were not closely associated with either (PsO, hypoPT, CMC).

Type 1 diabetes mellitus and Hashimoto's thyroiditis are the most common component diseases in the juvenile cohort

While the age of first onset of APS was not clear in 3.7% of cases, the majority of individuals had their first symptom after the age of 18, and only 20.5% of them had their first symptom before the age of 18. We hypothesised that childhood APS-component disease combinations may differ from those of adulthood. Regardless of the order of onset of the component diseases, the most common combination in those with the first childhood manifestation of APS was T1D with HT, accounting for 19.2% of cases. It is noteworthy that both T1D and HT were present in at least 50% of the multiple combinations. When looking at the adult cohort, the most common scenario was the co-presence of HT and RA. However, there were also a number of cases where HT was associated with T1D, CeD or AD.

The first manifestation is likely to influence the development of subsequent disorders in APS

An interesting pattern becomes apparent when looking at the five most commonly observed organ systems affected after the first manifestation. Thyroid disorders (HT and GD) showed a unique composition in terms of subsequent organ systems affected compared to the other three first

manifestations studied (T1D, AD, CeD). In patients diagnosed with HT or GD, gastrointestinal (32.8% and 40.8%, respectively) and systemic (31.9% and 32.7%, respectively) diseases had a high prevalence in the latter stages. In contrast, after T1D, AD and CeD, the endocrine system was predominantly affected (68.3%, 65.8% and 74.3%, respectively) in the later manifestations, with a lower but still relevant gastrointestinal (24.4%, 18.4% and 14.3%, respectively) and systemic (6.1%, 7.9% and 8.6%, respectively) involvement. The observed variation in this pattern showed a strong and statistically significant difference.

By following the course of the disease as each patient progressed through APS, significant pathways were observed. Interestingly, the pattern described above was also observed in this case: the composition of subsequent disease associated with HT or GD differed from that associated with T1D, AD or CeD. HT as the first manifestation could be followed by a wide variety of diseases, which occurred in approximately similar proportions, with CeD being the most frequent (16.8%). SS, RA and AIG were observed with a prevalence of more than 10% in these patients. As with HT, CeD was the most common secondary manifestation (21.4%) in patients with GD, while RA was the second (16.7%), AD the third (9.5%) and CeD the fourth (9.5%) most commonly acquired disease. Patients with T1D as the initial symptom developed HT in 52.1% of cases, which was strikingly higher than any other condition presenting as a secondary manifestation, such as GD (18.5%) or CeD (16.9%). A significant proportion of AD patients also developed HT (64.3%), making it the most common secondary manifestation, followed by GD (14.3%). When CeD was the primary symptom, similar proportions were observed, with HT being the most common (55.6%) and GD the second most common (25.9%) secondary manifestation. In those cases where T1D or CeD was the primary symptom, no neurological disease was observed. Two cases where the primary manifestation could not be determined were excluded from this analysis. The table below summarises which second manifestation usually follows each disease based on our own data. This shows that a screening algorithm for all diseases is not necessary. A screening protocol focusing on the most likely diseases and detailed information to patients on the symptoms and recognition of additional diseases may be sufficient.

Table 3. Frequency of the most frequent first manifestations and subsequent second manifestations in our own patient population

First disease	More manifestations				Total	
HT	CeD 17%	SS 12%	RA 11%	AIG 11%	T1D 7%	58%
DG	CeD 21%	RA 17%	AD 10%	CD 10%	SLE 7%	65%
T1D	HT 52%	DG 18%	CeD 17%			87%
AD	HT 64%	DG 14%				78%
CeD	HT 56%	DG 26%				82%

HT and GD are probably the two cornerstones of APS among the component diseases

Diseases and combinations of diseases are represented in a dimensionally reduced space to facilitate understanding of local and global connectivity. As in the network analysis results, HT and GD are clearly separated. Interestingly, the group of patients with a combination of HT and T1D is depicted as even more distinct from other combinations containing HT, leading to further speculation about the underlying factors. The mapping of the number of disease associations suggests that the apparently cohesive clusters, which are more separated from the background, are mainly composed of combinations of two diseases. When we compared this with the most frequent associations in our data, we found that the 11 combinations we identified as the most frequent form these clusters. Furthermore, it can be seen that these clusters of cases do not fully coincide with the APS categories. Although the clustering scheme is somewhat similar between the two methods, the current clinical classification of APS does not recognize the association between a specific condition and HT or GD. This can be observed, for example, in APS category III/a, where the classification is determined by the presence of T1D. Similarly, the majority of APS III/b is composed of patients with CeD, regardless of the association of HT or GD. However, as shown in our analysis, patients in APS III/a, and III/b subgroups clearly tend to be bifurcated according to their association with HT and GD.

3.3.C. Conclusions

APS is a complex, rare or considered rare disease. According to the literature, its prevalence is between 1 in 100 000 and 1 in 20 000. However, our data suggest that it is much more common, occurring in 12% of the 3180 patients with endocrine autoimmune disease in our database. Our study is one of the largest patient population studies on APS. The observed prevalence of the disease was unexpectedly high considering previously published international data, raising the question of the true prevalence of the disease. However, our clinic is a tertiary centre, which may introduce selection bias by including more complicated endocrine cases. In addition, the study was conducted in a single centre. The results are only indicative of the current status of the patients, without additional follow-up information. Unfortunately, the specificity of the diseases made it challenging to determine the initial manifestation

of each disease in many cases. Consequently, some data could not be analysed in relation to the first and second manifestations.

In this study, however, we introduced an analysis strategy that focused entirely on the associations of each disease, which deviated from the traditional patient classification method and which has not been used before to assess the development of APS.We found 28 different autoimmune diseases with 113 combinations, but more than half of the patients belonged to the 10 most common associations. More than two-thirds of patients had HT, making it the most common disorder.

Our network analysis and dimensional reduction visualization using the UMAP algorithm did not confirm the commonly used classification of APS for APS II, III and IV (analysis of APS I was not possible due to the low number of cases). The current clustering of the syndrome partially overlaps with the coherent clusters visualized on UMAP. This is to be expected, as both APS categories and clusters are defined on the basis of diseases and their co-occurrence. Our analysis confirmed that HT and GD are cornerstones of a network of cumulative autoimmune diseases and revealed a specific association of HT and T1D as a partially distinct subgroup. However, further studies are needed to define the APS categories more precisely. The current grouping focuses on the prevalence of a particular disease rather than its association with one of the two cornerstones (HT or GD). Exploring the genetic background may be the most useful information.

As for the first manifestations, an interesting finding was that T1D was much more likely to present as an initial disease than as a later manifestation. This phenomenon has so far only been studied separately in the APS IV subgroup. However, in our study it affects the whole population.

The age at APS release in our study was consistent with previous literature. Furthermore, we obtained remarkable results regarding the age of first symptom onset, especially in relation to gender. This observation adds new information to existing research. While it was previously known that women are more affected by autoimmunity, no previous study has shown that men have an earlier onset of APS, especially in AD, when it first manifests. The time between diagnosis of the first two manifestations varied widely. New autoimmune diseases may develop after 50 years from the first symptom, highlighting the importance of following up these patients, but making it challenging to establish a predictive algorithm.

Patients with AITD were classified as HT or GD, so the two thyroid diseases did not occur simultaneously in the same patient. Our primary aim in the network analysis was to assess the association of component abnormalities. We found that the similarities between HT and GD comorbidities were significantly more striking than the differences. We found almost complete overlap between the associated conditions, raising questions about the precise pathophysiological origins of these diseases. However, taking into account the individual associations, dimensional reduction mapping also effectively distinguished HT and GD patients.

CeD was another common disease, along with T1D, observed in the juvenile group. The prevalence of CeD is increasing worldwide. Recently, there have been several studies on the protective effect of a gluten-free diet (GFD) in certain autoimmune diseases

RA is also thought to be a disease influenced by the microbiome, suggesting that diet and infectious diseases may also influence its development. Interestingly, the most common dual combination in the adult cohort was HT+RA, followed by HT+T1D and HT+CeD.

A significant finding of our study is that the pattern of secondary manifestations differed significantly from that of T1D, AD or CeD in cases where thyroid autoimmunity was the initial symptom, and that

thyroid autoimmunity is often associated with gastrointestinal and systemic manifestations. It is also shown that many of the other most common diseases co-occur with HT or GD. Tracking the course of the disease after the most common first manifestations revealed the likely subsequent symptoms. HT was followed as the first manifestation by CeD, SS, RA and AIG, all of which occurred at a frequency of more than 10%. Among the most common secondary manifestations, GD was followed by CeD, RA, AD and CD. It is noteworthy that HT developed in more than 50% of patients with T1D, while GD and CeD were also common secondary manifestations, with more than 15% of cases each. Both CeD and AD patients had a high incidence of HT (more than 60% of CeD patients and more than 50% of AD patients), with GD being the second most common pathway. Detecting all of these is not the same as screening patients continuously, often unnecessarily. Among these diseases are several where antibody screening is not the first choice. For example, in GD, patients should be made aware of alarming symptoms, and in T1D, regular home fasting blood glucose monitoring is recommended. A short leaflet with at least the 10 most common manifestations should be considered. These could be given to patients with autoimmune disease by their general practitioner or family doctor, thus enabling them to carry out the first step of screening themselves. Of course, most diseases require specific laboratory tests, measuring antibodies. The question is whether there is a causal biological basis for their co-occurrence or whether they are simply combined by their frequency. Extending the study to a larger number of cases may provide a more comprehensive picture of the network of co-morbidities and facilitate targeted follow-up according to the disease of origin.

There have been several publications in the literature that have investigated the polymorphisms of HLA and non-HLA genes underlying certain autoimmune diseases in affected populations. These have only been able to identify what is the common predisposing factor in each autoimmunity, but unfortunately not what it predisposes to (which specific disease). If we want to predict autoimmunity later in life, it is absolutely necessary to elucidate the genetic background for prediction. Unfortunately, this was not possible in the present sample, but in the future it will definitely be necessary to perform appropriate genetic tests as a means of clarifying the combinations of diseases.

In general, APS is much more common than the literature suggests. The introduction of screening and follow-up systems focusing on additional autoimmune co-morbidities is crucial for patients with autoimmune disease. Up-to-date registries are essential to accurately assess the prevalence of the disease.

4. Clinical relevance of the work

At the start of our work (in 2016), we had a total of 64 patients in the clinic. We now have nearly 400 patients in our database. This does not mean that the number of APS patients has skyrocketed in the last 8 years. This huge difference demonstrates that it is a common but hidden, rarely diagnosed disease. Both the literature search and our own research have proven that these patients are numerous but difficult to find. One reason for this is that each autoimmunity is treated as a separate disease, often by different specialists. Another reason is the confusion of terminology. It is difficult to diagnose a patient without a uniform agreement on which group a given manifestation belongs to.

Our meta-analysis has highlighted the literary void and the difficulties of clustering. It also provided evidence for the key role of two autoimmune thyroid diseases in the disease.

We have tried to fill the gaps in our own patient material. We have created a network mapping between emerging diseases that can predict the sequential emergence of diseases, establish a sequence between them and highlight the most common manifestations. I think we have achieved part of our goal. We've identified the 10 most common, potentially fatal, interrelated autoimmune diseases, we've confirmed the key role of thyroid diseases. It also highlighted the interconnected network of diseases. These all provide tools for specialists - be they endocrinologists, immunologists, gastroenterologists, any specialist with autoimmune patients - to follow up patients, perform appropriate screening tests at the right intervals and establish the right diagnostic staging.

Our work does not end here. An even clearer picture, an even more efficient algorithm could be created after immunological and genetic testing of patients. Moreover, as we are now only seeing a snapshot of a clinic, following up patients and creating appropriate registries would make the fog around autoimmune polyglandular syndromes even clearer.

5. New clinical findings

1) APS is much more common than expected from previous data
2) Classification of patients into APS groups is difficult due to gaps in group definitions
3) HT and GD are cornerstones of a network of cumulative autoimmune diseases
4) HT and T1D, as a partially distinct subgroup, show a special association
5) Disease groups in APS III are too diverse to form a single category (APS III)
6) T1D is much more likely to present as an initial disease than as a later manifestation
7) Men have an earlier onset of APS, especially in AD, if it is the first manifestation
8) We found almost complete overlap between HT and GD-associated conditions, raising questions about the precise pathophysiological origins of these diseases
9) The pattern of secondary manifestations was significantly different from that of T1D, AD or CeD in cases where thyroid autoimmunity was the initial symptom
10) Thyroid autoimmunity is often associated with gastrointestinal and systemic manifestations
11) HT as first manifestation followed by CeD, SS, RA and AIG in >10% of cases
12) Among the most common secondary manifestations, GD was followed by CeD, RA, AD and CD
13) HT in >50% of T1D patients and GD and CeD as second manifestations in >15% of cases
14) >60% for CeD, >50% after AD for HT as a second disease

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