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# PITUITARY AUTOANTIBODIES IN ENDOCRINE DISORDERS

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***TO MY FAMILY***

## ABSTRACT

Autoimmune endocrine disorders are characterised by the development of autoantibodies to specific autoantigens in the target organs. Lymphocytic hypophysitis (LyH) is a disease characterised by inflammation of the pituitary gland, often resulting in hypopituitarism. The aetiology of LyH is considered to be autoimmune. However, only a few pituitary autoantigens have so far been identified. Reliable autoantibody markers are requested in the diagnostic procedure of LyH to avoid unnecessary surgical intervention.

The aim of this study was to evaluate the occurrence of pituitary autoantibodies in patients with nonadenomatous pituitary disease and also to identify novel pituitary autoantigens.

Autoantibodies to human pituitary cytosolic proteins were determined by immunoblotting. Reactivity to a  $M_r$  49 000 pituitary autoantigen was significantly more frequent in patients with idiopathic pituitary hormone deficiency (6/21 (28%)  $p < 0.05$ ) as well as their relatives (10/35 (28%)  $p < 0.02$ ) compared to control subjects (3/44 (6.8%)). The importance of these pituitary autoantibodies as markers for LyH remains to be confirmed.

Autoantibodies against human pituitary cytosol and ten additional organ-specific autoantigens were measured in sera from 30 patients with empty sella syndrome (ESS). None of the autoantibodies tested was more frequently found in ESS patients compared to healthy controls. Thus, by analysing autoantibodies we did not find evidence of ESS being associated with any autoimmune disease.

Autoantibodies to a novel 36-kDa pituitary cytosolic autoantigen were more common in patients with ACTH-deficiency (12/65 (18.5%) compared to control subjects (2/57 3.5%) ( $p < 0.0214$ ). In addition, autoantibodies to thyroglobulin (TG) were positively correlated to immunoreactivity against the 36-kDa pituitary autoantigen.

A human pituitary cDNA expression library was successfully constructed. Immunoscreening identified secretogranin II as a pituitary autoantigen in a patient with partial pituitary insufficiency and empty sella.

By immunohistochemistry, autoantibodies against ACTH and gonadotrophs were detected in sera from patients with autoimmune polyendocrine syndrome type 1 (APS1) and GH-deficiency. Sera from these patients also showed staining of monoamine and GABA nerve terminals in the pituitary intermediate lobe, in agreement with immunoreactivity towards enzymes involved in the biosynthesis of neurotransmitters. By screening of the human pituitary cDNA expression library we identified TDRD6 as a major autoantigen in APS1.

*Key words:* Lymphocytic hypophysitis, hypopituitarism, empty sella syndrome, APS1, autoantibodies, autoantigen, Secretogranin II, TDRD6.

# LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I. **Strömberg S**, Crock P, Lernmark Å, Hulting A-L. Pituitary autoantibodies in patients with hypopituitarism and their relatives. *Journal of Endocrinology* 1998;157:475-480.
- II. **Bensing S**, Rorsman F, Crock P, Sanjeevi C, Ericson K, Kämpe O, Brismar K, Hulting A-L. No evidence for autoimmunity as a major cause of the empty sella syndrome. *Experimental and Clinical Endocrinology & Diabetes* 2004;112:231-235.
- III. **Bensing S**, Kasperlik-Zaluska AA, Czarnocka B, Crock PA, Hulting A-L. Autoantibodies against pituitary proteins in patients with adrenocorticotropin-deficiency. *European Journal of Clinical Investigation* 2005;35:126-132.
- IV. **Bensing S**, Hulting A-L, Höög A, Ericson K, Kämpe O. Lymphocytic hypophysitis: Report of two biopsy-proven cases and one suspected case with pituitary autoantibodies. *To be submitted*.
- V. **Bensing S**, Fetissoff S, Perheentupa J, Gustafsson J, Husebye ES, Oscarson M, Ekwall O, Crock PA, Hökfelt T, Hulting A-L, Kämpe O. Pituitary autoantibodies in autoimmune polyendocrine syndrome type 1. *To be submitted*.

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## LIST OF ABBREVIATIONS

AADC	Aromatic-L-amino acid decarboxylase
ACTH	Adrenocorticotrophic hormone
APS1	Autoimmune polyendocrine syndrome type 1
BLOTTO	Fat-free skimmed milk
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ESS	Empty sella syndrome
FSH	Follicle-stimulating hormone
GAD/GAD65	Glutamic acid decarboxylase
GH	Growth hormone
IA-2	Islet cell antigen/protein tyrosine phosphatase 2
IGF-1	Insulin-like growth factor I
IgG	Immunoglobulin G
ITT	<i>In vitro</i> transcription and translation
LH	Luteinizing hormone
LyH	Lymphocytic hypophysitis
MIC	Thyroid microsomes
PBS	Phosphate-buffered saline
PGSF 1a	Pituitary gland specific factor 1a
PGSF 2	Pituitary gland specific factor 2
PRL	Prolactin
RNA	Ribonucleic acid
SCC	Side-chain cleavage enzyme
SDS	Sodium dodecyl sulphate
TDRD6	Tudor domain containing protein 6
TG	Thyroglobulin
TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase
TPO	Thyroid peroxidase
TRAK	TSH receptor antibodies
TSA	Tyramide signal amplification
TSH	Thyroid-stimulating hormone
17-OH	17 $\alpha$ -hydroxylase
21-OH	21-hydroxylase



# 1 INTRODUCTION

## 1.1 Autoimmune endocrine disease

The main purpose of the immune system is to defend us from invading agents like bacteria and viruses. To achieve this, this system must be able to discriminate an intruder from its own host. Several cell types and organs participate in this strictly regulated procedure. In autoimmune diseases the immune system attacks its own host, resulting in tissue damage or dysfunction.

Autoimmune disorders can be either *organ-specific* e.g. type 1 diabetes mellitus or *systemic* e.g. systemic lupus erythematosus. A common feature of autoimmune disease is the presence of *autoantibodies* directed against host tissue structures-*autoantigens*. Examples of autoantigens include intracellular enzymes, cell surface receptors and nucleic acids (Riley 1995, Song & Maclaren 1996). Autoantibodies have proven to be valuable markers for many autoimmune disorders and are regularly used as diagnostic tools.

Endocrine organs are common targets of autoimmune attacks. Isolated endocrine organs may be affected but often a number of autoimmune endocrine manifestations appear in one and the same individual, e.g. APS.

APS1 is a rare autosomal recessive disorder caused by mutations in the *AIRE* gene. This gene has recently been shown to promote the expression of organ-specific autoantigens in the thymus (Anderson et al 2002, Liston et al 2003). The classical clinical features of APS1 are hypoparathyroidism, Addison's disease and chronic mucocutaneous candidiasis (Betterle et al 1998). In addition, other organ-specific autoimmune components such as gonadal failure, type 1 diabetes mellitus and pituitary failure as well as ectodermal manifestations are found with variable penetrance. The classical definition of APS2 includes Addison's disease in combination with hypopituitarism and/or type 1 diabetes mellitus (Neufeld et al 1981). However, this is a genetically complex syndrome with a multifactorial aetiology, nowadays proposed to include other manifestations in various combinations (Peterson et al 2005).

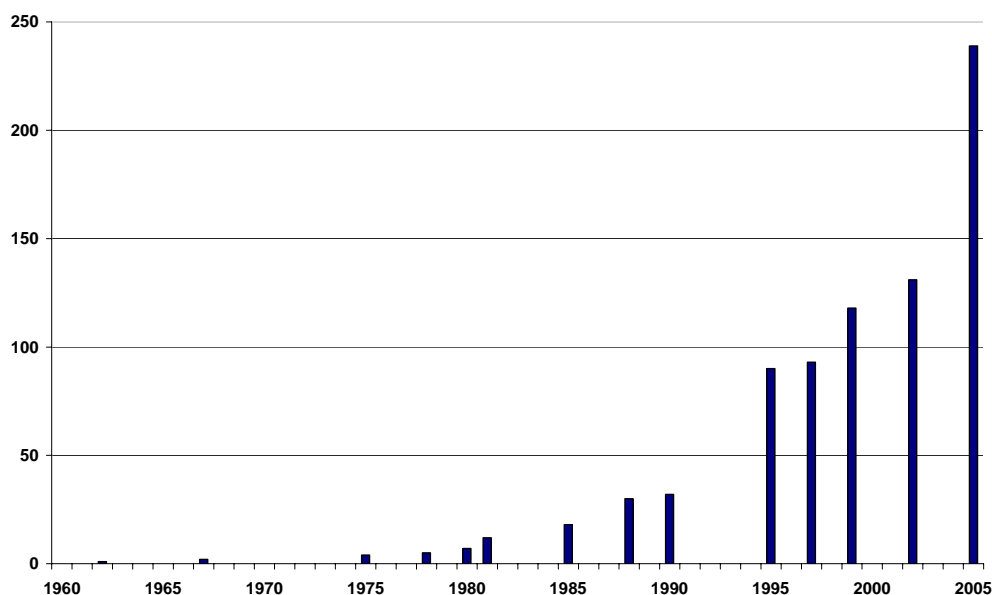
Anterior pituitary hormonal failure is often caused by pituitary tumours, cranial irradiation, surgery, trauma, infarction due to pituitary apoplexy or sarcoidosis. However, in some patients other causes of the pituitary dysfunction must be considered. It is believed that the pituitary, like other endocrine organs, may be the target of an autoimmune process. This condition is usually referred to as LyH.

## 1.2 Lymphocytic hypophysitis (LyH)

### 1.2.1 Historical notes

LyH was first described in 1962 (Goudie & Pinkerton 1962). Since then LyH has been reported with increasing frequency and today the English scientific literature includes over 230 biopsy-proven cases (Caturegli et al 2005). Earlier LyH diagnoses were all based on histopathological findings from autopsies. The first ante mortem diagnoses of LyH were published in 1980 (Mayfield et al 1980, Quencer 1980). With the recent introduction of MRI the number of patients with suspected LyH, i.e. non biopsy-proven cases, has also increased considerably (Beressi et al 1999).

*Accumulated number of reported patients with biopsy-proven LyH*



### 1.2.2 Pathological findings

The definition and diagnosis of LyH are both still based on histopathological findings on pituitary samples from surgery or autopsy.

#### *Macroscopic findings*

The affected pituitary gland may be of normal size, enlarged or atrophic (Cosman et al 1989). The colour of the gland varies from white-gray to yellow. It often appears firm with a tough texture and adherent to the sella turcica wall (Skandarajah et al 2002).

#### *Microscopic findings*

Lymphocytic infiltration of the anterior pituitary lobe is an uncommon finding in consecutive autopsy material and is a clearly pathological finding (Shanklin 1951). LyH is characterised by extensive chronic inflammation of the anterior pituitary gland. The posterior pituitary lobe and pituitary stalk are typically spared but may be involved (Koshiyama et al 1994, Unluhizarci et al 2001). The inflammatory infiltrate consists mainly of a polyclonal mixture of T and B lymphocytes, to a lesser extent accompanied

by plasma cells and macrophages (Tashiro et al 2002). The subsets of T lymphocytes have a CD4:CD8 ratio between 1:1 and 2:1 (Crock 1997, Gutenberg et al 2005). Other immunological cells like eosinophils, neutrophils and mast cells have also been observed. (Thodou et al 1995, Vidal et al 2002). Edema and fibrosis are commonly found but necrosis is rare. No granulomas or micro-organisms should be present. There are no reports of immune complex deposits or vascular injury. The severe inflammatory process causes various degree of tissue destruction, ranging from loss of a selective type of hormone secreting cells to more diffuse pituitary damage (Cosman et al 1989). The ACTH- and TSH-producing cells are known to be particularly vulnerable to the inflammatory process (Hashimoto et al 1997, Tashiro et al 2002).

#### *Electron microscopy findings*

Lymphocytes and plasma cells have been described adjacent to damaged adenohypophysial cells (Jensen et al 1986). Some of these pituitary cells showed signs of degeneration (Asa et al 1981).

### **1.2.3 Clinical spectrum**

LyH shows a striking female preponderance with a female: male ratio of 6 (Caturegli et al 2005). More than half of the female cases are related to late pregnancy or the postpartum period (Hashimoto et al 1997, Crock 1997). LyH has almost exclusively been diagnosed in adults, although occasionally also in childhood (Cemeroglu et al 1997). Females tend to present at a younger age (34.5 years) than males (44.7 years) (Bellastella et al 2003).

The classical clinical presentation of LyH is peripartum hypopituitarism, often accompanied by a pituitary mass mimicking a pituitary adenoma (Feigenbaum et al 1991, Thodou et al 1995). Common symptoms are headache (53%) and visual disturbances (40%) due to compression of the optic chiasm (Hashimoto et al 1997). If the pituitary mass involves the cavernous sinus, extraocular palsy may be present (Leung et al 2004). LyH may have a very rapid onset, and pituitary apoplexy has been described (Dan et al 2002).

Most LyH patients (78%) present with symptoms of hypopituitarism (Hashimoto et al 1997). One distinctive feature of LyH is the predilection for corticotroph destruction and isolated ACTH deficiency represents the most common isolated type of anterior pituitary hormone deficiency in LyH (Jensen et al 1986, Crock 1997). Unrecognised ACTH-deficiency has been the major cause of mortality in LyH patients. TSH-deficiency is also frequently seen. This is in contrast to the findings in patients with non-functioning pituitary adenomas, where the secretion of ACTH and TSH are often affected late in the course of disease. Even though ACTH- and TSH-producing cells seem especially vulnerable to the inflammatory process, it is important to remember that the hypopituitarism seen in LyH patients covers the spectrum from any hormonal deficiency to panhypopituitarism.

Hyperprolactinemia is reported in 23% of LyH cases (Caturegli et al 2005). This is a normal finding during late pregnancy and the lactation period. However, in LyH patients elevated PRL levels have also been found in elderly women as well as in men sometimes causing galactorrhea and sexual dysfunction (Ezzat & Josse 1997). It has been speculated that the hyperprolactinemia seen in LyH may be a direct effect of PRL stimulating antibodies (Bottazzo et al 1975), or alternatively it may be secondary to compression of the pituitary stalk with loss of inhibitory factors or massive destruction of lactotrophs.

Diabetes insipidus may appear if the LyH involves the posterior pituitary lobe or causes compression of the posterior pituitary or pituitary stalk (Iglesias & Diez 2002).

If present, this is a sign distinguishing LyH from a non-functioning pituitary adenoma, which is rarely associated with diabetes insipidus.

LyH is frequently (20-25%) associated with autoimmune disorders of both organ-specific and systemic nature (Beressi et al 1999). Hashimoto's thyroiditis is most commonly seen (Patel et al 1995, Barbaro & Loni 2000). Other reported associations include Graves' disease (Bayram et al 1998), Addison's disease (Sobrinho-Simoes et al 1985), type 1 diabetes mellitus (Hashimoto et al 1997), atrophic gastritis (Mazzone et al 1983), APS 1 (Ward et al 1999), systemic lupus erythematosus (Ji et al 2000), Sjögren's syndrome (Li et al 1999) and primary biliary cirrhosis (Nishiki et al 1998).

The natural history of LyH is unclear but the disease is thought to progress through different stages. In the acute inflammatory phase the pituitary can be enlarged.

The LyH may be clinically evident due to symptoms of a pituitary mass and/or acute hypopituitarism. However, the disease may also be subclinical with vague signs and symptoms.

In the sub acute phase of LyH, the pituitary inflammation sometimes spontaneously resolves (McGrail et al 1987, Ozawa & Shishiba 1993). The pituitary can appear slightly enlarged or normal on MRI. Symptoms like headache and visual defects may disappear and the pituitary hormonal function recover. Successful pregnancies after resolved LyH have been reported (Brandes & Cerletty 1989, Gagneja et al 1999), but so is recurrence of disease (Nishioka et al 1997).

The pituitary inflammatory process commonly does not resolve, and the gland is gradually destroyed and replaced by fibrosis. Hypopituitarism then becomes manifest and the patient will depend on life long hormonal replacement therapy. The pituitary may be of normal size or atrophic, presenting as an empty sella at this chronic stage of LyH (Ishihara et al 1996, Ruelle et al 1999).

#### **1.2.4 Radiological features**

The radiological images of LyH vary depending on stage and extent of the inflammatory process (Cosman et al 1989). Despite the introduction of MRI in the evaluation and diagnosis of hypothalamic-pituitary diseases, LyH with pituitary enlargement is easily mistaken for a pituitary adenoma (Sautner et al 1993, Leung et al 2004). LyH should therefore always be considered as a possible diagnosis of a pituitary mass. Radiological findings, suggestive of LyH are summarised in Table 1.

In its chronic form, LyH may show a normal pituitary on MRI or signs of pituitary atrophy and empty sella (Mau et al 1994). Several radiological signs are suggestive of LyH, but not specific enough. Histological examination is therefore still required to fully establish the diagnosis.

**Table 1: Radiological features suggestive but not specific of LyH.**

	<b>Radiological features of LyH</b>	<b>References</b>
Pre contrast pituitary appearance	Glandular homogeneity Symmetric enlargement	Caturegli et al 2005
Post contrast pituitary appearance	Intensely homogeneously enhancing mass Adjacent enhancing dura mater Enhancement confined to the periphery of the mass Delayed contrast enhancement	Ahmadi et al 1995 Saiwai et al 1998 Lee et al 1994 Sato et al 1998
Posterior pituitary lobe	If affected, loss of the hyperintense bright spot	Imura et al 1993
Pituitary stalk	Thickened but not displaced	Abe et al 1995
Sella turcica floor	Flat and intact	Bellastella et al 2003

### **1.2.5 Treatment/Follow up**

The management of LyH is not established. Unrecognised LyH with hypopituitarism is potentially fatal (Gal et al 1986). Therefore, a fast correct diagnosis of hypopituitarism and adequate hormonal replacement therapy are crucial.

Many physicians today advocate a conservative approach regarding treatment of the pituitary mass, which may resolve spontaneously (Patel et al 1995, Krimholtz et al 2001). The patient is then monitored clinically with repeated hormonal assessments, visual field examinations as well as MRI.

Some patients require active treatment due to mass symptoms. Standard therapy then consists of transphenoidal resection of the mass or oral administration of corticosteroids. The first case report on successful glucocorticoid treatment of LyH was published in 1994 (Beressi et al 1994), and today it is proposed by many authors to be the first line of therapy. One prospective trial of high dose methylprednisolone pulse therapy has been performed with promising results (Kristof et al 1999).

In cases with severe or progressive visual impairment, surgical intervention is sometimes necessary (Kidd et al 2003). Surgery should also be considered if glucocorticoid treatment has been unsuccessful in reducing a large pituitary mass, and also when the patient shows recurrence of symptoms (Reush et al 1992). Good relief of visual defects and headache is usually reported after surgery. However, permanent damage of pituitary hormonal function is also usually observed (Stelmach & O'Day 1991, Buxton & Robertson 2001).

A few LyH patients have received alternative treatments including use of low dose stereotactic radiotherapy (Selch et al 2003), methotrexate (Tubridy et al 2001) or azathioprine (Lecube et al 2003) with good results.

### 1.2.6 Aetiology

From the first case report in 1962, LyH has been proposed to have an autoimmune origin (Goudie & Pinkerton 1962). A disease is commonly referred to as autoimmune based on certain criteria: direct proof, indirect evidence and circumstantial evidence (Rose & Bona 1993).

#### 1. *Direct proof*

Direct proof requires that autoreactive autoantibodies and/or T cells can transmit the disease from human to human or human to animal. In neonatal myasthenia gravis autoantibodies transmit the disease from the mother to the child across the placenta. However, most disorders considered to be autoimmune, including LyH, lack this kind of direct proof.

#### 2. *Indirect evidence*

Indirect evidence requires isolation of self-reactive T cells and/or autoantibodies from the target organ, or the establishment of animal disease models. Reproducible pituitary lymphocytic infiltration has been evoked in animals by several scientists. Levine reported the first successful model of experimentally induced LyH (Levine 1967). He injected pituitary extract and Freud's adjuvant into mice resulting in lymphocytic infiltration of the pituitary. Experimental LyH has later been induced in rhesus monkey (Beck & Melvin 1970), rabbits (Klein et al 1982) as well as hamsters (Yoon et al 1992).

In the study by Yoon et al (1992), pituitary autoantibodies could be detected but they could not induce LyH by passive transfer. It was instead shown that neonatal thymectomy prevented LyH development, thus indicating that T cells are important for disease induction.

Polyendocrinopathy, including type 1 diabetes mellitus and experimental LyH, has been reported in mice infected with reovirus type 1 (Onodera et al 1981). In addition to pituitary lymphocytic infiltration, autoantibodies against insulin and GH were detected in this model. However, no clear relationship between LyH and GH autoantibodies could be seen (Onodera et al 1982).

In 2001, experimental LyH was induced in female Lewis rats by injections of homologous pituitary homogenate and complete Freud's adjuvant (Watanabe et al 2001). No severe lymphocytic infiltration of the anterior pituitary gland was seen, but GH, TSH and LH were identified as major autoantigens.

Recently, it has been shown in a transgenic mouse model that the pituitary gland is susceptible to CD8 T-cell-mediated autoimmunity, triggered by viral infection, and resulting in LyH, GH deficiency and dwarfism (De Jersey et al 2002, De Jersey et al 2004).

#### 3. *Circumstantial evidence*

A striking female preponderance as well as the association with other autoimmune diseases support an autoimmune aetiology of LyH (Jacobson et al 1997).

The histopathological findings resemble those of other autoimmune endocrine disorders (Muir & Maclaren 1991). In addition, LyH patients have shown responsiveness to immunosuppressive treatment (Kristof et al 1999, Tubridy et al 2001, Lecube et al 2003).

Several autoimmune diseases are known to have cycles of remission and relapses. They are also often influenced by pregnancy (Kovacs & Olsen 1999). The association between LyH and pregnancy is well documented (Baskin et al 1982). Furthermore, a severe form of LyH has been described in pregnant mice (Levine 1967). Autoimmune diseases are often associated with particular alleles of the major histocompatibility complex (Ermann & Fathman 2001), but data from LyH patients are so far scarce and inconclusive (Ezzat & Josse 1999, Bellastella et al 2003).

Autoimmune endocrine disorders are characterised by the development of autoantibodies directed against autoantigens in the target organ(s). Some of these autoantibodies have proven to be valuable diagnostic disease markers (Baekkeskov et al 1990, Winqvist et al 1992). A few pituitary autoantigens have been identified. The existence of pituitary autoantibodies has been established in patients with LyH. However, reliable autoantibody markers are requested in the diagnostic procedure of LyH to avoid unnecessary surgical intervention. Publications regarding pituitary autoantibodies and pituitary autoantigens are summarised below.

### **1.2.7 Pituitary autoantibodies**

Several techniques and tissue substrates have been used for the detection of pituitary autoantibodies, see Table 2.

#### *Complement consumption test*

Pituitary autoantibodies were first detected by Engelberth & Jezkova in 1965. They used a complement consumption test, with homogenised human pituitaries from autopsies as antigen substrate. The frequency of pituitary autoantibodies was investigated in 128 healthy women during pregnancy and the post-partum period. Eighteen % of the females developed pituitary autoantibodies post-partum and a few of them later showed signs suggestive of hypopituitarism. Unfortunately, no hormonal data were collected to verify pituitary hormonal dysfunction. The complement consumption test is today considered to have both low sensitivity and specificity.

#### *Indirect Immunofluorescence test*

The indirect immunofluorescence test was developed by Bottazzo et al in 1975 and is still widely used. Many researchers report on the difficulties in measuring pituitary autoantibodies, and the importance of choosing the right pituitary substrate. Normal human fresh frozen material from surgery or fetal pituitaries are considered to be ideal substrates (Gluck & Scherbaum 1990) but the supply of such tissue is strictly limited. Instead, primate, non-primate as well as different pituitary cellines have been used with variable results.

Immunoreactivity against various kinds of pituitary hormone secreting cells have been detected e.g. lactotrophs (Bottazzo et al 1975), somatotrophs (De Bellis et al 2005), thyrotrophs (Pouplard 1982), gonadotrophs (Pouplard et al 1985) and corticotrophs (Scherbaum et al 1987, Sauter et al 1990); but often only in low titres.

The presence of pituitary autoantibodies has been established in only 16/58 LyH patients (Caturegli et al 2005). Moreover, the specificity seems poor since pituitary immunoreactivity has also been found in patients with various autoimmune diseases

without pituitary hormonal deficit, such as Type 1 diabetes mellitus (Mirakian et al 1982), APS (Bottazzo et al 1975), Hashimoto's thyroiditis and Grave's disease (Kobayashi et al 1988, Hansen et al 1989). In addition, pituitary autoantibodies have also been reported in patients with disorders of not clearly autoimmune or non autoimmune origin such as idiopathic hypopituitarism (Sugiura et al 1986), ESS (Komatsu et al 1988), cryptorchidism (Pouplard et al 1985) and pituitary adenomas (Scherbaum et al 1987, Komatsu et al 1988).

Recently it has been suggested that pituitary autoantibodies directed against guinea pig somatotrophs, when detected at high titres, may be considered as good diagnostic markers of autoimmune GH-deficiency in adults (De Bellis et al 2003, De Bellis et al 2005).

In addition to immunoreactivity against anterior pituitary autoantigens, autoantibodies to antidiuretic hormone secreting cells have been described to be associated with idiopathic central diabetes insipidus in patients below 30 years of age (Pivonello et al 2003).

### *Immunoblotting*

Crock et al introduced the immunoblotting method for pituitary autoantibody detection in 1993. Human and animal autopsy substrates seem to yield satisfactory results, thus overcoming the need for fresh pituitary tissue. With this methodology, multiple pituitary autoantigens can be detected and characterised by their molecular weight. The autoantigens can also be localised to the cytosolic or membrane fraction. On the other hand, the cell type to which the autoantibodies bind can not be identified.

Autoantibodies against a 49-kDa pituitary cytosolic protein were demonstrated in high frequencies among patients with biopsy-proven LyH (7/10, 70%), suspected LyH (12/22, 55%) and various autoimmune endocrine diseases (8-42%) compared to 20% of patients with pituitary adenomas and 9.6% of healthy controls (Crock 1998). Also, reactivity against a 40-kDa pituitary cytosolic autoantigen was detected in 50% of patients with biopsy-proven LyH compared with only 7.7% of healthy controls. The 49-kDa pituitary autoantigen has later been identified as enolase (O'Dwyer et al 2002a,b) but contradictory data on the importance of enolase autoantibodies as markers for neuroendocrine autoimmunity have been published (Tanaka et al 2003). The 40-kDa pituitary autoantigen remains to be identified.

Immunoreactivity against a 22-kDa pituitary cytosolic autoantigen has been detected in patients with diabetes mellitus (Kobayashi et al 1997, Yabe et al 1998), suspected LyH as well as idiopathic pituitary insufficiency (Kikuchi et al 2000, Takao et al 2001). The 22-kDa protein has been identified as GH (Takao et al 2001) but the importance of GH autoantibodies as markers for LyH needs to be established.

Autoantibodies against not yet identified human pituitary membrane proteins have been detected in a few sera from patients with LyH (Nishiki et al 2001). These immunoreactivities were reported to be specific for LyH, but were relatively insensitive.

### *Enzyme-linked immunosorbent assay (ELISA)*

An ELISA method was developed in 1998 for pituitary autoantibody detection, using rat pituitary as antigen (Yabe et al 1998). Significantly higher levels of pituitary autoantibodies have since been detected in patients with type 1 diabetes mellitus (Yabe et al 1998), autoimmune thyroiditis (Nishino et al 2001) and various pituitary disorders



(Kikuchi et al 2000) compared to normal controls. There are no reports of sera from biopsy-proven LyH patients having been tested with this methodology.

**Table 2: Techniques and substrates used for detection of pituitary autoantibodies.**

<b>Technique</b>	<b>Pituitary substrate</b>		<b>References</b>
<b>Complement consumption</b>	Human	Autopsy material	Engelberth & Jezkova 1965
<b>Indirect Immunofluorescence</b>	Human	Fetal glands	Scherbaum et al 1987 Gluck & Scherbaum 1990 Gluck et al 1993
		Fresh material from surgery	Bottazzo et al 1975 Mirakian et al 1982 Gluck et al 1993
		Autopsy material	Bottazzo et al 1975 Pouplard et al 1985
	Primate pituitary glands	Baboon	De Bellis et al 2003 De Bellis et al 2005
		Rhesus monkey	Maghnie et al 1994 Maghnie et al 1995
		Cynomolgous monkey	Gluck & Scherbaum 1990
	Non primate pituitary glands	Rat	Bottazzo et al 1975 Pouplard et al 1980 Sugiura et al 1986 Kobayashi et al 1988 Kajita et al 1991
			Guinea pig
		Bovine	Bottazzo et al 1975
		Porcine	Hansen et al 1989
	Cellines	Murine AtT <sub>20</sub>	Sugiura et al 1987 Komatsu et al 1988 Kajita et al 1991
		Rat GH <sub>3</sub>	Sugiura et al 1987 Komatsu et al 1988 Kajita et al 1991
<b>Immunoblotting</b>	Human	Membrane fraction	Crock et al 1993 Nishiki et al 2001
		Cytosolic fraction	Crock et al 1993 Crock 1998 Takao et al 2001 Goswami et al 2002
	Non primate homo-genized pituitaries	Rat	Yabe et al 1995 Yabe et al 1998 Kikushi et al 2000 Nishino et al 2001
			Porcine
<b>ELISA</b>	Human	Pituitary adenoma cells	Keda et al 2002
	Non primate	Rat	Yabe et al 1998 Kikuchi et al 2000 Nishino et al 2001 Keda et al 2002
<b>ITT and immunoprecipitation of pituitary proteins</b>	Human		Tanaka et al 2002a Tanaka et al 2003 Tatsumi et al 2003
<b>Immunoscreening of pituitary cDNA expression library</b>	-	-	not previously reported

*In vitro* transcription and translation (ITT) and immunoprecipitation of pituitary proteins

In 2002, [<sup>35</sup>S]-radiolabeled GH, PGSF1a and PGSF2 were expressed by ITT and immunoprecipitated with patients' sera (Tanaka et al 2002a). PGSF1a and PGSF2 are two recently identified pituitary specific proteins of unknown function (Tanaka et al 2002b). Autoantibodies against one or more of these three antigens were detected in subgroups of patients with LyH, idiopathic hypopituitarism as well as various autoimmune diseases but not in patients with non-functioning pituitary adenomas. The authors suggested that these antibodies could be useful for the diagnosis of LyH. However, no follow-up studies of these pituitary autoantibodies have so far been published.

Recently, the same research group reported that autoantibodies against *in vitro* expressed [<sup>35</sup>S]-radiolabeled alpha-enolase were present in sera from LyH patients (Tanaka et al 2003). This is in agreement with previous findings (Crock 1998). However, Tanaka et al also detected alpha-enolase autoantibodies in sera from patients with non-functioning pituitary adenomas, in a similar frequency as in LyH patients, indicating that these autoantibodies are probably not suitable as diagnostic markers for LyH.

The search for reliable autoantibody markers in patients with LyH has been in progress for forty years. However, the significance of pituitary autoantibodies as well as their relation to LyH is not yet understood. With new techniques e.g. immunoblotting and cDNA expression libraries the possibilities to detect pituitary autoantibodies and characterise pituitary autoantigens have increased.

## 2 AIMS OF THE STUDY

The overall aim of this thesis was to evaluate the occurrence of pituitary autoantibodies in patients with nonadenomatous pituitary disease.

The specific aims were

- To assess the frequency of pituitary autoantibodies in patients with idiopathic pituitary deficiency and their relatives.
- To investigate a possible autoimmune origin of the empty sella syndrome (ESS).
- To evaluate the occurrence of pituitary autoantibodies in patients with isolated ACTH-deficiency.
- By construction of a human pituitary cDNA expression library, create the possibility of identifying novel pituitary autoantigens.
- To establish and identify pituitary autoantigens in patients with autoimmune polyendocrine syndrome type 1 (APS1), by the use of immunohistochemistry and screening of our pituitary cDNA expression library.

### 3 MATERIALS AND METHODS

#### 3.1 Immunoblotting (paper I, II, III)

Normal human pituitary tissue was obtained at autopsy, 4-8 h post mortem and stored at  $-70^{\circ}\text{C}$ . The pituitaries were homogenized in PBS with protease inhibitors (leupeptin, pepstatin, phenylmethylsulfonylfluoride, ethylenediamine tetraacetate and ethyleneglycol tetraacetate) and centrifuged at  $400 \times g$  and then  $100\,000 \times g$  to give a cytosolic fraction. The cytosol was depleted of IgG using protein A Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) and stored in aliquots at  $-70^{\circ}\text{C}$ . The cytosol preparations were then boiled in the presence of 1,4-Dithiothreitol and fractionated on  $10 \times 10,5$  cm SDS-polyacrylamide gels (10% running gel, 4% stacking gel) by electrophoresis. A total amount of  $50 \mu\text{g}$  protein was loaded in each well. Pre-stained molecular weight markers (Bio-Rad, Hercules, CA) were included in each experiment. After electrophoresis, separated proteins were transferred by wet blotting to polyvinylidene difluoride transfer membranes (Bio-Rad paper I, NEN Life Science Products, Boston, MA, paper II,III). After blocking for 1 h in 5% BLOTTO/PBS, membranes were divided into ten lanes using a Deca-Probe Incubation Manifold (Hoefer, San Francisco, CA). Lanes were incubated with patient, relative or control serum diluted at 1:50 in 1% BLOTTO/PBS on a shaker at  $4^{\circ}\text{C}$  overnight. Serum from a patient previously shown to have strong immunoreactivity against a 49-kDa pituitary autoantigen was used as a positive control in each experiment. Negative control lanes were incubated with 1% BLOTTO/PBS only. Membranes were then washed first with PBS-0.05% Tween 20 and then with PBS, followed by incubation with alkaline phosphatase conjugated anti-human IgG antiserum (for details, see Table 3) in 1% BLOTTO/PBS for 1-1.5 h at room temperature on a shaker. After final washing, membranes were incubated with 5-bromo-4chloro-3indolyl phosphate and nitroblue tetrazolium (NEN Life science Products) until colour development was optimal. Results of the immunoblotting assay experiments were reported by observers who were blinded to the status of the serum samples

**Table 3: Alkaline phosphatase conjugated anti-human IgG antisera.**

Paper I	goat anti-human IgG 1:2500	Sigma-Aldrich Inc, St Louis, MO
Paper II	sheep anti-human IgG 1:4000	Amrad Biotech, Victoria, Australia
Paper III	sheep anti-human IgG 1:4000	Sigma-Aldrich Inc, St Louis, MO

### 3.2 Construction and screening of a human pituitary cDNA expression library (paper IV, V)

A cDNA expression library was constructed from 5 µg of human pituitary gland Poly A<sup>+</sup> RNA (Clontech, Palo Alto, CA) using the ZAP Express® cDNA synthesis kit and ZAP Express® cDNA Gigapack® III Gold cloning kit (Stratagene Cloning Systems, La Jolla, CA). The library, containing  $1.7 \times 10^6$  unique cDNA clones, was then amplified once.

Sera from two patients with hypopituitarism (paper IV) (diluted 1:100) and two APS1 patients with GH deficiency (paper V) (diluted 1:1000 and 1:3000) were used for immunoscreening of the library as previously described (Rorsman et al 1995). In brief, we plated 50 000 plaque forming units of the amplified library on E coli XL-1. After 3.5 hours of culture at 42 °C, plates were overlaid with nitrocellulose filters (Hybond C, Amersham) previously soaked with isopropyl β-D-thiogalactopyranoside (IPTG) and cultured for another 3.5 hours at 37 °C. The filters were removed from the plates and washed in 20 mM Tris-HCL, pH 7.5/0.1% gelatin/0.05% Tween-20 (TBS-GT). Non-specific protein binding was blocked with 1% gelatin in 20 mM Tris-HCL, pH 7.5, for 1 hr, after which the filters were washed in TBS-GT. The filters were then blocked with normal goat serum diluted in TBS-GT for 1 hour to reduce unspecific binding of the secondary antibody. After repeated washing, filters were incubated overnight with sera from the screening patients diluted in TBS-GT. The next morning, filters were washed in TBS-GT and incubated with alkaline phosphatase-conjugated goat anti-human IgG diluted 1:3000 in TBS-GT for 1.5 hours. After final washings, membranes were incubated with 5-bromo-4chloro-3indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) until colour development was optimal. Positive clones were rescreened until pure isolates were obtained.

*In vitro* excision of pBK-CMV phagemid vectors from the ZAP express vector were performed according to the manufacturer's protocol. Isolated cDNA clones were analysed by 5' and 3' sequencing using a dye-terminator-sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and ABI 377 or 3700 sequencers (Perkin-Elmer Applied Biosystems, Foster City, CA). The sequence data were compared to available databases using the Basic Local Alignment Search Tool (Altschul et al 1997). In paper V, the cDNA clone encoding TDRD6 was completely sequenced by primer walking with internal primers (CyberGene, Huddinge, Sweden).



*Filter with a mixture of positive and negative clones*

### **3.3 *In vitro* transcription and translation (ITT) of autoantigens and immunoprecipitation (paper II, V)**

Recombinant [<sup>35</sup>S]-radiolabeled 21-OH, 17-OH, SCC, AADC, TPH, TH, GAD, IA-2 and TDRD6 were produced by ITT in TnT® coupled reticulocyte lysate systems (Promega, Madison, WI). The correct sizes of the radioactive enzymes were verified by SDS-polyacrylamide gel electrophoresis (BioRad, Richmond, CA). [<sup>35</sup>S]-Methionine incorporation was measured by trichloroacetic acid precipitation, followed by scintillation counting.

Microtiter plates (96 wells) with filter bottoms (MABV N12, Millipore, Bedford, MA) were used for immunoprecipitation experiments. After preincubation with a buffer containing 150 mM NaCl, 20 mM Tris-HCL and 0.02% NaN<sub>3</sub> (pH 8.0) (buffer A) for 1 hour, the wells were coated with 1% BSA (Sigma chemicals, St Louis, MO) in buffer A for 2 hours. The plate was then washed twice with 0.05% Tween-20 in buffer A and finally, once with 0.1% BSA and 0.15% Tween-20 in buffer A (buffer B). The [<sup>35</sup>S]-radiolabeled autoantigen was mixed with patient or blood donor serum (1:20 dilution) in buffer B and incubated overnight at 4 °C on a shaker. The mixtures of sera and autoantigen were then transferred to the wells, and a 50% slurry of protein A-Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) in buffer B was added to each sample. The plate was shaken on a rotating platform for 45 min at 4°C and washed three times in buffer B using a vacuum manifold. After drying, scintillation fluid was added and the plate counted in a Microbeta counter (Wallac Oy, Turku, Finland). All serum samples were run in duplicates. Patients with known high titers of autoantibodies against the autoantigens were used as positive controls. Blood donor sera served as negative controls in paper II and 4% BSA in paper V.

The GAD65 and IA-2 assays in paper II have been validated by participation in the international workshops for the standardization of islet antibody determination, and the upper levels of normal were 0.09 for the GAD65 assay and 0.064 for the IA-2 assay. The remaining autoantibody detection results were expressed as an index [(cpm sample-cpm negative control)/ (cpm positive control-cpm negative control) x 100]. The upper normal limit of the autoantibody index was set to the mean value for blood donors plus 3 standard deviations except for TDRD6 analysis, where an arbitrary upper normal limit of the autoantibody index was set to 30 as this value separated the APS1 cohort into those with clearly elevated values and those with normal values.

### **3.4 Analysis of thyroid autoantibodies (paper II, III)**

*Paper II:* TPO autoantibodies were determined using DYNOfest® anti-TPO<sub>n</sub>, a competitive radioimmunoassay with enzymatic active native TPO from human thyroids as antigen (BRAHMS Diagnostica GmbH, Berlin, Germany). Results were expressed as U/ml of TPO autoantibodies and the upper level of normal was 60 U/ml. TRAK autoantibodies were determined by TRAK-assay®, a radioimmunoassay with TSH receptor isolated from porcine thyroid as antigen (BRAHMS Diagnostica GmbH, Berlin, Germany). TRAK values were expressed as U/l and the upper level of normal was 9 U/L.

**Paper III:** All patients' sera were tested for autoantibodies against TG and MIC (Thymune T and Thymune M, respectively, Murex Diagnostics, Dartford, UK) (normal values: up to 1/120).

For the detection of autoantibodies against TPO, highly purified human TPO was coated on polystyrene plates. The plates were then washed, coated with BSA, washed again and filled with various dilutions of patients' sera. After 1 h incubation at room temperature, unbound material was removed by extensive washing. Antibodies bound to coated antigen were detected using affinity-purified anti-human antibodies conjugated with horseradish peroxidase. The absorbance with chromagen was measured with an ELISA scanner. Pathological values: equal or greater than 1/800. For further details see Kasperlik-Zaluska et al 1994.

### 3.5 Immunohistochemistry (paper IV, V)

**Paper IV:** Pituitary tissue samples from two patients were fixed in formalin and embedded in paraffin. Hematoxylin/eosin was used as a routine staining and immunohistochemistry was performed with the Avidin Biotin Complex method according to the manufacturers' instructions (Vectastatin, Vector, Burlingame, CA). Primary antibodies against ACTH, GH, PRL, lymphocytes and plasma cells were purchased from DakoCytomation, CA.

**Paper V:** Sera from six APS1 patients diagnosed with GH deficiency and ten healthy blood donors were selected for immunohistochemistry. Experiments were designed in accordance to guidelines on animal care. Male and female guinea pigs (wt 250-300 g) (B & K Universal, Stockholm, Sweden) were housed under controlled environmental conditions with a constant light-dark cycle (light on between 6.00 and 18.00), a temperature of 21-22°C and a relative humidity of 40-50%; food and water were given ad libitum. Animals were anaesthetized with sodium pentobarbital (0.15 mg/100 g body weight, i.p.) and perfused via the ascending aorta with Tyrode's Ca<sup>2+</sup>-free solution at 37°C, followed by a mixture of 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer (pH 6.9, 37°C) (Zamboni & De Martino 1967) and then by the same, but ice-cold mixture. The pituitaries were rapidly dissected out, immersed in the same fixative for 90 min and rinsed with 10% sucrose in 0.1 M phosphate buffer (pH 7.4) overnight. Tissue was snap-frozen using dry ice. Fourteen-µm thick pituitary sections were cut on a cryostat (Microm, Heidelberg, Germany) and thaw-mounted on chrome alum-gelatin-coated glass slides.

The TSA immunohistochemical technique (Adams 1992) was used for single labeling. Incubation with APS1 or control sera (diluted 1:2000-1:10000) overnight at 4°C was followed by horseradish peroxidase-conjugated, rabbit antihuman IgG (1:200, Dako A/S, Copenhagen, Denmark) using the TSA-Plus Fluorescein System (PerkinElmer Life Science, Inc. Boston, MA). For double-labeling, the TSA technique was followed by conventional immunohistochemistry (Coons 1958) with rabbit antiserum against LH (1:400, Biogenesis, Poole, England), guinea pig antiserum against PRL (1:1000, UCB Bioproducts, Alleud, Belgium), goat antiserum against TSH (1:1000, Biogenesis), sheep antiserum against GH (1:1000, Biogenesis) or mouse antiserum against ACTH (1:1000, Peninsula Laboratories, Belmont, CA). Secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) conjugated with Rhodamine Red

were used at (1:40-1:80) dilution. The specificity of the binding was tested by preadsorption of human sera (diluted 1:2000-1:4000) with synthetic peptides ( $10^{-5}$ - $10^{-8}$  M), LH, FSH (Sigma Chemicals) or ACTH (Bachem, Bissendorf, Switzerland) as well as 60 000-120 000 cpm [ $^{35}$ S]-radiolabeled GAD, AADC, TH or TPH expressed *in vitro*.

Sections were mounted in a mixture of glycerol and 0.1 M phosphate buffered saline (3:1), pH 7.4, containing 0.1% para-phenylenediamine (Sigma) as anti-fading agent (Platt & Michael 1983). After processing, the sections were examined in a Bio-Rad Radiance Plus confocal laser scanning system (Bio-Rad, Hemel Hemstead, U.K.) installed on a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) with objectives lenses x10, x20 and oil immersion x60. Single 0.5  $\mu$ m thick optical planes were scanned for single and double labeling. Digital images resulting from the microscopy were optimised for image resolution, and images with double-labeling were merged in Adobe PhotoShop 6.0 (Adobe Systems Incorporated, San Jose, CA).

### **3.6 Statistical analyses (paper I, II, III, V)**

Statistical analyses were performed using Statistica, StatSoft Inc, Tulsa, OK.

**Paper I:** Reactivity to pituitary cytosolic proteins was compared between groups using Fisher's exact test. A *p*-value of less than 0.05 was considered statistically significant.

**Paper II:** Fisher's exact test was used in the statistical analyses when comparing the immunoreactivity to eleven different autoantigens in patients vs. control subjects. A *p*-value of less than 0.05 was considered statistically significant.

**Paper III:**  $\chi^2$ -test with Yate's correction was used when comparing immunoreactivities to pituitary autoantigens in patients vs. control subjects. The two-tailed Fisher's exact test was used when comparing clinical and immunological parameters in patients with positive pituitary immunoreactivity vs. patients with negative pituitary immunoreactivity. Statistical significance was set at  $p < 0.05$ .

**Paper V:** Two-tailed Fisher's exact test was used to compare the frequencies of different disease manifestations in APS1 patients with and without TDRD6 autoantibodies. A *p*-value of less than 0.05 was considered statistically significant.



## 4 RESULTS AND DISCUSSION

### 4.1 Pituitary autoantibodies in patients with hypopituitarism and their relatives (paper I).

Autoantibodies to human pituitary cytosol proteins were determined by immunoblotting in sera from patients with idiopathic hypopituitarism (n=21).

The cohort of patients comprised 11 men and 10 women and the age range was 24-87 years. Sera from 35 family members (15 men and 20 women) (first degree relatives and husband and wife) and 44 healthy control subjects (28 men and 16 women) were also tested for pituitary autoantibodies.

Six patients suffered from panhypopituitarism while 13 were diagnosed with different types of isolated pituitary deficiencies. The remaining two patients had partial hypopituitarism with preserved PRL and GH secretion, respectively. Diabetes insipidus was seen in one of the patients with panhypopituitarism.

Coexisting autoimmune disease was reported in 4/21 patients and four additional patients had records on various detectable autoantibodies in sera.

In 18/21 patients CT or MRI had been performed. Eight patients exhibited normal findings, while ten patients had pathological findings consisting mainly of cisternal herniations (6/10). No patient had an enlarged sella turcica, neither was a pituitary tumour suspected in any case.

Reactivity to a  $M_r$  49 000 protein was significantly more frequent in patients (6/21 (28%)  $p<0.05$ ) and also in relatives (10/35 (28%)  $p<0.02$ ) compared with controls (3/44 (6.8%)). A number of other pituitary cytosolic proteins were detected by individual sera, but there were no significant patterns or differences between patients, relatives and controls.

**Comments:** The clinical features of the patients were compared with the clinical data on LyH patients recorded in the literature. LyH is known to affect predominantly females with a female:male ratio of 6 (Caturegli et al 2005). We did not see any female predominance in our patient cohort. In addition, LyH seems to be strongly correlated to pregnancy but our patient cohort contained only two women who developed hypopituitarism in relation to pregnancy. Most biopsy-proven LyH patients present with enlarged pituitary masses, often in combination with visual disturbances (Powrie et al 1995). None of the patients in our study had a pituitary mass on radiological examination or any visual symptoms.

LyH is thought to progress through different stages. In the acute phase, the pituitary is often enlarged, but later the gland may be more or less destroyed by the severe inflammation. Cisternal herniation may therefore represent an end stage of LyH. Three of the  $M_r$  49 000 antibody-positive patients in our study showed cisternal herniation, and this was also the most common pathological finding in the total patient group. The patients in our study had isolated or partial hormone deficiency as well as total panhypopituitarism, which is also reported for patients with biopsy-proven LyH (Jensen et al 1986, Cosman et al 1989).

Autoantibodies are known to be present among relatives of patients with organ-specific autoimmune disease. However, most of these relatives do not develop any autoimmune disorder, perhaps due to the fact that a combination of certain genetic markers and environmental factors are needed for disease development. This might also be the case for autoimmune pituitary disease and may explain the absence of hypopituitarism in relatives with pituitary autoantibodies in our study. Autoantibodies to non-characterised rat pituitary cytosolic proteins have previously been described in a few family members of two patients with Sheehan's syndrome and one patient with partial hypopituitarism (Kajita et al 1991).

Autoantibodies to the  $M_r$  49 000 pituitary protein has been reported in sera from 7/10 (70%) patients with biopsy-proven LyH and in 4/20 (20 %) of patients with pituitary adenoma (Crock 1998). It was therefore proposed that these autoantibodies could be markers for LyH and helpful in the differential diagnosis of pituitary masses. In 2002 the  $M_r$  49 000 autoantigen was identified as alpha-enolase, an enzyme in the glycolytic pathway (O'Dwyer et al 2002a). Today we know that autoantibodies against alpha-enolase are not LyH specific but common markers of many organ-specific as well as systemic autoimmune diseases (Gitlits et al 2001, Fujii et al 2005). Moreover, high frequency of alpha-enolase autoantibodies has been reported in patients with pituitary adenomas. Thus,  $M_r$  49 000 autoantibodies are probably not suitable for specific diagnosis of LyH (Tanaka et al 2003). Future epitope mapping of the alpha-enolase may reveal if sera from LyH patients target different regions compared to sera from pituitary adenoma patients and healthy controls.

## **4.2 No evidence for autoimmunity as a major cause of the empty sella syndrome (paper II).**

In this study, we measured eleven different organ-specific autoantibodies in sera from 30 patients with ESS and 50 healthy subjects to evaluate possible autoimmune components in ESS.

The mean age of the patients (25 women and 5 males) was 55.5 years (range 33-80) and the duration of disease ranged from a few weeks to 30 years. No patient exhibited a cisternal herniation that was less than 30% of the sellar volume at diagnosis.

Neurological symptoms and signs like headache, dizziness, and fatigue were frequently found among patients. A striking feature of the patient cohort was that half of them suffered from type 2 diabetes mellitus (11/30) or impaired glucose tolerance (4/30). Primary hypothyroidism was found in 9/30 (30%) patients and pituitary dysfunction in only 4/30 (13%).

Sera from six patients reacted against a 49-kDa pituitary cytosolic protein. In addition, three patients showed immunoreactivity to TPO and three other patients towards GAD. No patient reacted to more than one autoantigen. Thus, the majority of the ESS patients (18/30) exhibited no immunoreactivity at all, in fact no immunoreactivity was found more frequently among ESS patients compared to healthy blood donors. We found no significant correlation between pituitary autoantibodies and the ESS patients' sellar size, pituitary hormonal dysfunction or any other immunoreactivity.

**Comments:** ESS has been proposed to be caused by chronic or intermittent increase of intracranial pressure (Brismar & Bergstrand 1981) as well as partial pituitary apoplexy (Wakai et al 1981, Bjerre et al 1986). Since ESS has been reported as an end stage of LyH, an autoimmune origin has also been suggested (Beressi et al 1999).

In contrast to some earlier studies (Komatsu et al 1988, Keda et al 2002), we did not find an increased incidence of pituitary autoantibodies in ESS compared to control subjects. There may be several explanations to this diversity in results, for example different methodologies (immunoblotting vs. immunofluorescence) and antigen sources (human pituitary cytosol vs. rodent cellines/adenohypophysis or human adenomatous tissue). We used human pituitary cytosol as antigen substrate and target autoantigens might be bound to pituitary cell membranes.

Notably, although statistically not significant, two of four (50%) ESS patients with hypopituitarism were found to have immunoreactivity against the 49-kDa pituitary protein. Furthermore, three of 11 (27%) with normal sellar size showed immunoreactivity to this protein compared to three of 19 (16%) with an enlarged sella. Studies on larger ESS patient cohorts are needed to establish possible associations between pituitary autoantibodies related to hypopituitarism and normal sella turcica size, respectively.

None of the other ten organ-specific autoantibodies tested were more frequently found in ESS patients compared to healthy controls. Thus, by analysing autoantibodies we did not find evidence of ESS being associated with any autoimmune disease.

### **4.3 Autoantibodies against pituitary proteins in patients with adrenocorticotropin-deficiency (paper III).**

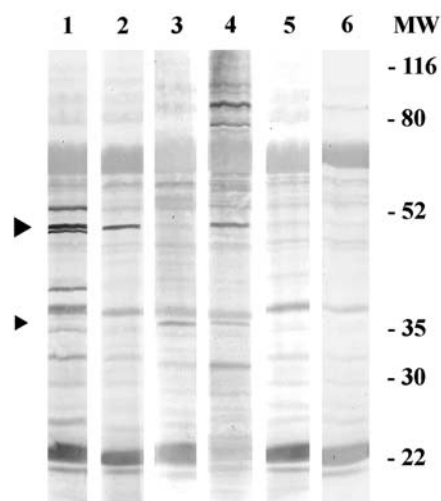
The occurrence of autoantibodies against human pituitary cytosolic proteins were evaluated by immunoblotting in a large group of patients with ACTH-deficiency (n=65) and healthy subjects (n=57). All sera were analysed in duplicate and the immunoreactivities were reported as negative, positive or strongly positive. In addition, the occurrence of pituitary autoantibodies was correlated to clinical features and immunological parameters of the patients.

Pituitary hormonal evaluation in patients revealed isolated secondary adrenal insufficiency in 61/65 patients and additional pituitary insufficiencies in four females. Coexisting autoimmune diseases were found in 33/65 patients (51%), of which 23 had one and 10 had two or more autoimmune disorders. Thyroid autoimmune diseases were strikingly most common. Therefore, in all patients' sera, autoantibodies against TG, MIC and TPO were examined. Thyroid autoantibodies were found in 55/65 patients. In 31 patients thyroid autoantibodies were present without laboratory signs of hypothyroidism.

Autoantibodies to a novel 36-kDa pituitary cytosolic autoantigen were detected in sera from 18.5% (12/65) patients compared to 3.5% (2/57) control subjects ( $p=0.0214$ ). When taking into account only those subjects with strongly positive results on both runs (six patients vs. two control subjects), the significance was lost. The group of patients with positive 36-kDa immunoreactivity had a higher frequency of TG autoantibodies ( $p=0.014$ ).

Immunoreactivity to a 49-kDa pituitary cytosolic autoantigen was observed in sera from 21.5% (14/65) patients and 8.8% (5/57) control subjects, without significant difference ( $p=0.0910$ ). In addition, we saw no differences in clinical and/or immunological parameters in patients with or without autoantibodies to the 49-kDa pituitary autoantigen.

Four patients showed immunoreactivity against both the 36-kDa and the 49-kDa pituitary autoantigens, while 43 patients did not exhibit autoantibodies against any of these two autoantigens.



***Immunoblotting membrane showing immunoreactivities against two pituitary autoantigens indicated by arrows***

**Comments:** An autoimmune cause of ACTH-deficiency has been proposed as it is known to be a characteristic feature of LyH. Autoantibodies against four identified pituitary autoantigens; neurone-specific enolase, GH, PGSF 1a and PGSF2 have been detected in a limited number of patients with isolated ACTH-deficiency (Crock 1998, O'Dwyer et al 2002a, Tanaka et al 2002a, Tanaka et al 2003).

In the present study we were able to identify a 36-kDa pituitary protein as a minor autoantigen in ACTH-deficiency. Our immunoblotting method does allow characterisation of pituitary antigens by molecular weight. Further studies to completely identify the 36-kDa pituitary autoantigen are needed, for example by screening of a pituitary cDNA expression library with sera from patients with ACTH-deficiency. To validate the use of autoantibodies against the 36-kDa pituitary autoantigen as markers for autoimmune ACTH-deficiency, the occurrence of these autoantibodies in different pituitary diseases needs to be evaluated. Also, the cut-off level for autoantibody titres in healthy subjects should be further elucidated.

The majority of ACTH-deficient patients in this study did neither show autoantibodies to the 36-kDa nor to the 49-kDa pituitary autoantigen. There is a possibility that the ACTH-deficiency seen in these patients does not have an autoimmune background with no autoantibodies in patients' sera to detect. However, it is also possible that we did not recognise additional pituitary autoantibodies in our immunoblotting experiments. For example, we used human pituitary cytosol as antigen substrate and target autoantigens might be bound to pituitary cell membranes. Furthermore, it is possible that some pituitary autoantibodies cannot recognise the denaturated form of antigen used in our immunoblotting assay and might be detectable by other methods. One should also keep

in mind that autoantibody titers may fluctuate and even disappear in the time course of an autoimmune disease (Nikolai et al 1987).

In conclusion, our findings of pituitary autoantibodies in patients' sera support the theory that an autoimmune destruction of corticotrophs may be one underlying cause of hormonal deficit in patients with ACTH-deficiency.

#### **4.4 Lymphocytic hypophysitis: Report of two biopsy-proven cases and one suspected case with pituitary autoantibodies (paper IV).**

We describe three patients who were evaluated at our clinic because of pituitary hormonal deficiencies.

Hormonal assessment of patient A (a 73-year old woman) revealed a mild secondary hypothyroidism, low gonadotropin levels, a slightly elevated PRL but normal GH-IGF-1 and cortisol values. Patient B (a 63-year old man) was found to have low TSH, cortisol and testosterone levels but PRL and GH-IGF-1 values were within normal limits.

Both patients had pituitary masses suggestive of a pituitary adenoma on MRI and transsphenoidal pituitary surgery was performed. Histopathological examinations revealed LyH in both cases.

Postoperatively, patient A had persistently low gonadotropins but all other pituitary hormones were within normal limits. No pituitary mass was seen at MRI postoperatively and an empty sella gradually developed. Four years postoperatively primary hypothyroidism and diet treated diabetes mellitus type 2 were diagnosed. Patient B developed panhypopituitarism postoperatively. The pituitary mass showed a spontaneous regression after the minor surgical resection but the pituitary stalk remained slightly thickened.

In addition, we report on a 79 year old man, patient C, where hormonal assessment revealed partial hypopituitarism with low TSH, cortisol and testosterone levels. CT showed no signs of a pituitary tumour but a pronounced empty sella without sellar enlargement, the reason why LyH was suspected.

A cDNA expression library was constructed from human pituitary gland Poly A<sup>+</sup> RNA. Screening of the human pituitary cDNA expression library with sera from patient B and C identified 14 positive clones. Nine clones identified by patient B did not correspond to any protein of pituitary or neuroendocrine specific interest. All five clones recognised by patient C serum encoded secretogranin II.

**Comments:** LyH should always be considered as a possible diagnosis of a pituitary mass. Patient A and B represent two cases of biopsy-proven LyH *not* seen in the classical relation to pregnancy or the post-partum period. The ACTH and TSH producing cells are known to be vulnerable to the inflammatory process (as seen in patient B) but all pituitary hormones may be affected (Hashimoto et al 1997, Tashiro et al 2002). LyH may be extremely difficult to distinguish from a pituitary adenoma. We did not suspect LyH in patient A and B until the observations at surgery and histopathological examination, the latter showing considerable lymphocytic infiltration of the anterior pituitary glands and damage to ACTH immunoreactive cells.

Postoperatively both patients' pituitary masses disappeared without any immunosuppressive treatment.

Secretogranin II was identified as a pituitary autoantigen in patient C. This secretory protein belongs to a group of acid proteins called chromogranins and is known to be commonly present in granules of neuroendocrine cells and in vesicles of some neurons (Rosa et al 1985, Wiedenmann & Huttner 1989). Secretogranin II is believed to contribute in the sorting and condensing of secretory proteins into secretory granules (Gerdes et al 1989). The protein is also thought to be a precursor of several biologically active peptides (Kirchmair et al 1993, Anouar et al 1998). In normal human pituitary tissue secretogranin II has been reported to be abundantly expressed, especially in gonadotrophs, thyrotrophs and corticotrophs but not in somatotrophs and lactotrophs (Vallet et al 1997). Since patient C selectively showed the corresponding pituitary insufficiencies, i.e. LH-FSH, TSH and ACTH-deficiency, we speculate on an autoimmune background to his disease. Further studies are needed to elucidate whether secretogranin II autoantibodies will be useful as diagnostic markers for LyH.

#### **4.5 Pituitary autoantibodies in autoimmune polyendocrine syndrome type 1 (paper V).**

In this study, we aimed to identify novel pituitary autoantigens in patients with APS1 by immunohistochemistry and immunoscreening of a human pituitary cDNA expression library.

Sera from 3/6 APS1 patients with isolated GH-deficiency showed immunostaining, one against ACTH and two against gonadotrophs, whereas no such staining was seen with sera from ten healthy controls.

Four out of six APS1 sera showed staining of a fiber-plexus surrounding the endocrine cells in the pituitary intermediate lobe. Preadsorption of sera with a mixture of the four enzymes GAD, AADC, TH and TPH completely abolished all fluorescent structures.

Our human pituitary cDNA expression library was screened with sera from two APS-1 patients with GH-deficiency. A protein encoded by a TDRD6 cDNA clone was identified as a major autoantigen in APS1. Positive immunoreactivity against *in vitro* translated TDRD6 fragments was shown in 42/86 (49%) APS1 patients, but not in any patients with LyH, Addison's disease, diabetes mellitus type 1, Sjögren's syndrome, systemic lupus erythematosus or in healthy controls.

**Comments:** Hypopituitarism is a minor APS1 manifestation but isolated as well as multiple pituitary insufficiencies have been described. Pituitary autoantibodies have been detected in a few APS1 patients, however not related to pituitary dysfunction (Bottazzo et al 1975, Betterle et al 1998).

Human pituitary material of desirable quality was not available for our immunohistochemistry experiments and we therefore had to use pituitary glands from animals. We used pituitaries from guinea pigs, since previous studies have shown good results with this species (Pouplard 1982).

ACTH autoantibodies have been detected in patients with eating disorders as well as in patients with various neuropsychiatric conditions and also in some healthy control subjects (Fetissov et al 2002, Fetissov et al 2003). Further studies are needed to clarify

whether ACTH autoantibodies may play a pathophysiological role in APS1 and stress-related disorders.

Our finding of immunoreactivity against gonadotrophs in two APS1 patients with GH-deficiency is in agreement with a recently published case report (Cocco et al 2005).

We were not able to identify the targeted gonadotroph autoantigen(s). Notably, these two APS1 patients exhibited primary ovarian failure. The disturbed GH release might be caused by damage to the hypothalamus rather than to the pituitary gland, since we did not detect any immunoreactivity against somatotrops.

The pituitary intermediate lobe is known to receive a dopaminergic (Baumgarten et al 1972, Björklund et al 1973) and a GABA-ergic ((Oertel et al 1982) innervation, both of a central origin. In fact there is evidence that these terminals at least in part are identical, in agreement with coexistence of TH and GAD in arcuate neurons (Everitt et al 1984). In addition, there is a central serotonergic innervation of the intermediate lobe (Friedman et al 1983) which should contain TPH. Serotonin can also be present in dopamine terminals, but in this case following uptake by the dopamine transporter (Vanhatalo et al 1994), i.e. these terminals do not contain TPH. Taken together, in the intermediate lobe there are presumably nerve terminals containing TH+ AADC (dopamine), TH+AADC+GAD (mixed dopamine-GABA), GAD alone and TPH alone. The adsorption result seems to reflect that distribution, GAD enzyme being most efficient in reducing staining.

We were also able to identify TDRD6 as a major autoantigen in APS1. This protein is known to be expressed mainly in the testis and only in very low levels in other endocrine tissues. The TDRD6 protein has not been studied much but it is known to contain seven tudor domains that initially was suggested to bind RNA (Ponting 1997). However, recent data suggest that tudor domains interact with proteins containing methylated arginines (Selenko et al 2001, Cote & Richard 2005).

Autoantibodies against TDRD6 were found to be highly APS1 specific. We were unable to find an association between TDRD6 immunoreactivity and any major APS1 manifestation. However, the lowest P value was seen when TDRD6 autoantibodies was correlated to gonadal failure ( $p=0.168$ ). Further studies may reveal, if TDRD6 immunoreactivity is correlated to other so far unrecognised APS1 manifestations.





## 5 CONCLUSIONS

- Immunoreactivity to a 49-kDa pituitary autoantigen was significantly more common in patients with idiopathic pituitary hormone deficiency as well as their relatives compared to control subjects.
- We found no evidence of empty sella syndrome (ESS) being associated with any specific autoimmune disease, by analysing pituitary autoantibodies as well as a large number of other organ-specific autoantibodies.
- Autoantibodies to a novel 36-kDa pituitary autoantigen were more frequently seen in patients with isolated ACTH-deficiency compared to control subjects. In addition, autoantibodies to thyroglobulin (TG) were positively correlated to immunoreactivity against the 36-kDa pituitary autoantigen.
- A human pituitary cDNA expression library was successfully constructed. Secretogranin II was identified as an autoantigen in a patient with partial pituitary insufficiency and empty sella.
- Autoantibodies against ACTH and gonadotrophs were detected in sera from patients with APS1 and GH-deficiency. Sera from these patients also showed staining of monoamine and GABA nerve terminals in the pituitary intermediate lobe, in agreement with immunoreactivity towards enzymes involved in the biosynthesis of neurotransmitters. TDRD6 was identified as a major autoantigen in APS1.

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