Addison’s Disease and Type 1 Diabetes Mellitus

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"Ἐν οἴδα, ὅτι οὐδὲν οἴδα"

“I know one thing; that I know nothing”

Socrates, “Plato’s Apology”, Athens, 399 BC
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ABSTRACT

Background: Patients with type 1 diabetes (T1DM) and patients with Addison’s disease (AD) need life-long replacement therapy with insulin and glucocorticoids (GCs), respectively. Both groups have reduced life-expectancy. Autoimmune polyendocrine syndrome combining T1DM and AD is rare and with very limited outcome data available. Patients with concurrent T1DM and AD comprise a treatment challenge due to the counter-balancing effects of insulin and GCs on glucose metabolism. In patients with diabetes, glycated haemoglobin is an excellent diagnostic and therapeutic biomarker. No such biomarker of GC action is available for patients with AD.

Aims: To study the epidemiology of patients with concurrent T1DM and AD. More specifically, to investigate the incidence and mortality in patients with T1DM and AD, and elucidate early indicators for AD development in this population. To discover putative biomarkers of GC action.

Methods: Population-based, real-world data were derived from six linked Swedish National Registries, including the National Diabetes Register. Depending on the research question, cases were matched to five control subjects: we determined AD incidence (T1DM vs general population), and early indicators and mortality (T1DM+AD vs T1DM). The main statistical methods used were: Cox regression analysis, analysis of covariance, estimated group proportions, and Kaplan-Meier survival curves. The biomarker study was a randomised, crossover study in patients with AD, where patients were studied during states of near-physiological GC exposure and GC withdrawal. Gene expression from peripheral blood mononuclear cells and circulating microRNAs and metabolites were integrated into a network analysis.

Results: The incidence of AD among patients with T1DM was 193 (95% CI: 152–245) per million patient-years. The risk of developing AD among patients with T1DM was 10.8 (95% CI: 7.1–16.5) times higher than in the general population. Prodromal signs for the development of AD in patients with T1DM were treatment for thyroid disease, infections requiring hospital admission,
multiple diabetic complications (retinopathy in particular), and rescue therapy for hypoglycaemia. Patients with concurrent T1DM and AD had 4.3 (95% CI: 2.6–7.0) times increased risk for death than patients with T1DM alone and died most frequently from diabetic complications. The biomarker study succeeded in generating two completely different states of GC exposure. Integration of gene expression data, miRNA and metabolomic data delivered a network model with modules of putative biomarkers of GC action.

Conclusions: The higher risk of AD among patients with T1DM and the higher mortality in patients with concurrent T1DM and AD indicate the need of an improved strategy for patient management. Finally, the experimental study identified novel, potential biomarkers of GC action for further validation.

Keywords: Addison’s disease, type 1 diabetes mellitus, glucocorticoids, incidence, early indicators, drug prescription, mortality, biomarkers.

**SAMMANFATTNING PÅ SVENSKA**

*Bakgrund:* Både personer med typ 1 diabetes mellitus (T1DM) och personer med Addisons sjukdom har ökad mortalitet. Båda sjukdomarna är autoimmuna och det är känt att förekomsten av en autoimmun sjukdom ökar risken för ytterligare en sådan. Att ha både T1DM & Addisons sjukdom är inte väl studerat, huvudsakligen på grund av att det är ovanligt. Denna kombination är svårbehandlad eftersom hormonerna involverade i sjukdomarna, insulin och kortisol, har motverkande effekter på glukosmetabolism. Insulin ersättning kan justeras med hjälp av en excellent biomarkör (HbA1c), men ingen sådan biomarkör finns för kortisol.


*Resultat:* Incidens av Addisons sjukdom bland personer med T1DM var 193 per million person-år (95% CI: 152–245). Personer med T1DM hade en 10.8 gånger (95% CI: 7.1–16.5) ökad risk av att drabbas av Addisons sjukdom jämfört med bakgrundspopulationen. De tidiga tecknen av Addisons sjukdom var: behandling mot tyreoidea sjukdom, inneliggande behandling för infektioner, multipla diabeteskomplikationer, diabetes retinopati, och akut behandling mot hypoglykemi. Personer med T1DM & Addisons sjukdom hade 4.3 gånger (95% CI: 2.6–7.0) ökad mortalitet jämfört med personer med T1DM. Den huvudsakliga dödsorsaken var diabeteskomplikationer. Baserad på mätningar av serum och urin GC:er, biomarkör studien lyckades generera två olika tillstånd av GC substitution. Sammanställningen av genexpression, microRNA samt metabolit data med nätverk analys indikerade att modellen var relevant för att finna markörer för GC effekt.

*Konklusion:* Den högre risken att utveckla Addisons sjukdom bland personer med T1DM samt den högre mortaliteten i T1DM & Addison sjukdom gruppen, betonar vikten av att upptäcka och optimalt behandla Addisons sjukdom hos personer med T1DM. Den experimentella studien har potential för upptäckter av framtida biomarkörer för GC effekt.
LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

Paper I:  **Incidence, prevalence, and seasonal onset variation of Addison’s disease among persons with type 1 diabetes mellitus: nationwide, matched, cohort studies**
*Eur J Endocrinol.* 2018;178:115-22

Paper II:  **Early clinical indicators of Addison’s disease in patients with type 1 diabetes mellitus: a nationwide, matched, observational, cohort study**
Chantzichristos D, Persson A, Miftaraj M, Eliasson B, Svensson A-M & Johannsson G
Manuscript

Paper III:  **Mortality in patients with diabetes mellitus and Addison's disease: a nationwide, matched, observational cohort study**
*Eur J Endocrinol.* 2017;176:31-9

Paper IV:  **Identification of down-stream biomarkers of glucocorticoid action in man using subjects with adrenal insufficiency as experimental model**
Manuscript
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ABBREVIATIONS

ACTH  Adrenocorticotropic hormone
AD    Addison’s disease
APS2  Autoimmune polyendocrine syndrome type 2
CI    Confidence interval
GC    Glucocorticoid
GC-MS Gas chromatography-mass spectrometry
HC    Hydrocortisone
ICD   International Classification of Diseases
LC-MS Liquid chromatography-mass spectrometry
LISA  Longitudinal Integration Database for Health Insurance and Labor Market Studies
miR/miRNA microRNA
NDR   National Diabetes Register
PBMC  Peripheral blood mononuclear cells
OPLS-DA Orthogonal Projections to Latent Structures Discriminant Analysis
SD    Standard deviation
SMD   Standardised mean difference
SNIR  Swedish National In-patient Register
T1DM  Type 1 diabetes mellitus
TSH   Thyroid-stimulating hormone

Manuscripts are prepared in accordance with approved gene nomenclature.
1 INTRODUCTION

This thesis studies, systematically and for the first time, the rare combination of two metabolic diseases, Addison’s disease (AD) and type 1 diabetes mellitus (T1DM). Previously, knowledge on the risk of developing AD in patients with T1DM and the prognosis of patients having both diseases was limited.

Advances in the care of patients with T1DM are remarkable and accelerating in contrast with those for AD. An important role in this progress is due to glycated haemoglobin (HbA1c), an excellent biomarker that reflects biochemical control and long-term prognosis. This thesis also includes an experimental study aimed at identifying putative biomarker(s) of glucocorticoid (GC) action.

1.1 GLUCOCORTICOIDS

Cortisol is the main endogenous GC in humans. Endogenous GC secretion from the adrenal glands is under tight dynamic control by the hypothalamic-pituitary-adrenal axis and is regulated in a circadian (24-h cyclical), pulsatile ultradian rhythm (with the amplitude of peaks decreasing during the course of the day) and larger pulses in the case of acute stress [1-4]. There is considerable evidence in animal models indicating that pulsatile circulating GC levels, via transient GC receptor activation, lead to transcriptional pulsing at a genetic level [5,6].

GCs act via the ubiquitously expressed GC receptor, belonging to the nuclear receptor superfamily. The tissue-specific responsiveness to GCs is regulated by the pre-receptor metabolism of GCs and the interaction of the GC receptor with either tissue-specific transcription factors (which regulate transcription of several thousand genes) or non-genomic factors [7-9]. As a result of this complexity, circulating levels of cortisol only loosely relate to tissue activity of cortisol and this is why serum cortisol has limited value as a biomarker for GC action [10,11]. Additionally, there is poor correlation between symptoms and serum cortisol levels [12].

GCs have a key role in the metabolic, vascular and immunological response to stress, and their effects are largely permissive, modulating responses to many other stimuli [13]. The metabolic effects of GCs are to promote lipolysis, proteolysis and gluconeogenesis. GCs stimulate the deposition of glycogen, increase hepatic glucose output, inhibit glucose uptake and utilisation in peripheral tissues, and lead to insulin resistance and increased plasma glucose levels [14,15]. The metabolic effects of GCs are more pronounced in the
evening than in the morning; elevated evening GC levels have been associated with glucose intolerance, abdominal obesity and coronary arteriosclerosis [16-18]. At the same time, the effects of GCs are highly diverse and there is wide inter-individual variation in sensitivity to them [19]. The detailed mechanistic pathways by which GCs act remain poorly defined.

GCs are essential for survival. Extreme excess (Cushing’s syndrome) or deficiency (AD) in humans causes striking clinical abnormalities and increased mortality [20,21]. More subtle changes in the action of GCs are thought to be important in the aetiology of many common diseases such as obesity, hypertension and type 2 diabetes mellitus [22].

The first synthesis of exogenous GC (11-deoxycortisone) took place in 1937 [23]. Another synthetic cortisone (compound E or Kendall’s compound) was first given to a patient with AD in 1948 [24]. Synthetic GCs have long been amongst the most commonly prescribed drugs as they have a central therapeutic role in lung disorders, rheumatic diseases, haematological malignancies and transplanted patients [25,26].

Metabolic side effects of synthetic GCs are common. Patients receiving oral GCs have an increased use of diabetic and antihypertensive drugs, increased risk of having osteoporotic fractures or cardiovascular events, and excess cardiovascular mortality [25,27-30]. The reasons behind these side effects are both the non-physiological dose and time-exposure profile [31,32]. A more individualised approach based on a biological response marker should improve outcome in all patients treated with synthetic GCs.

1.2 ADDISON’S DISEASE

Pathogenesis

In AD or primary adrenal insufficiency, the adrenal cortex has impaired production and secretion of GCs (cortisol), mineralocorticoids (aldosterone) and adrenal androgens [33]. In developed countries, autoimmune AD (predominantly with positive 21-hydroxylase autoantibodies) accounts for about 85% of cases [34]. Up to 60% of patients with autoimmune AD have an additional autoimmune condition, most frequently thyroid disease (50%) or T1DM (12%), which constitutes autoimmune polyendocrine syndrome type 2 (APS2) [35-37].

Autoimmune AD is often characterized by an insidious onset leading to overt disease after a varying period of time [38]. In the natural history model of autoimmune AD, genetic susceptibility (predominantly specific HLA haplotypes) is underlying, which is followed by unknown triggering factors.
leading to progressive destruction of the adrenal cortex. Elevated plasma renin activity is an initial sign followed by the appearance of 21-hydroxylase autoantibodies and decreased peak cortisol levels (following a synthetic adrenocorticotrophic hormone [ACTH] stimulation test); finally, serum ACTH is increased and basal cortisol decreased before progression to overt clinical disease, in which ACTH is greatly increased and both peak stimulated and basal cortisol are greatly decreased [39].

Epidemiology

The prevalence of AD in different European populations varies between 93 and 221 per million and has an estimated incidence of 4.4–6.2 per million per year. In common with several other autoimmune disorders, prevalence and incidence have been rising (Table 1) [35,36,40-44]. Women are more frequently affected than men and age at diagnosis peaks between 30 and 50 years of age [35,40-42].

*Corresponding 95% CI presented when available.
†The prevalence of both AD and T1DM (49 among 426 patients with AD) was 20 per million patients.

AD is an inevitably fatal disease in the absence of treatment [45]. Even when AD is diagnosed and treated, it is associated with increased risk of premature death, mainly due to cardiovascular diseases, cancer, infections, sudden death and adrenal crisis [21,36,46].
Diagnosis

The clinical presentation of AD depends on the pace and extent of the loss of adrenal function. Diagnosis is delayed because of non-specific symptoms and signs. A cross-sectional study found that 20% of the patients suffered for more than 5 years before being diagnosed and two-thirds of all patients with AD presented to medical professionals three or more times with symptoms of adrenal failure before a correct diagnosis was made [47]. Patients affected with autoimmune AD display a wide spectrum of clinical presentations and severity, often displaying a prolonged, insidious onset [48]. AD may also present with an adrenal crisis, which often occurs in the context of a stressful event (such as infection or trauma) or in the case of bilateral adrenal infarction, haemorrhage or pituitary apoplexy.

As symptoms of AD are non-specific, a high level of clinical suspicion is required to make a correct diagnosis. Common features of AD include weight loss, anorexia, nausea, vomiting, lethargy, hypoglycaemia, hyperpigmentation of skin and mucosal surfaces, and fatigue (in up to 95% of patients) [38]. In addition, postural hypotension, muscle cramps, abdominal discomfort and salt craving can arise due to mineralocorticoid insufficiency. In women, loss of axillary and pubic hair can arise due to lack of androgens.

Apart from signs and symptoms, altered drug use is reported before the diagnosis of autoimmune AD with increased prescription of gastrointestinal, anti-anemic, thyroid and lipid-modifying drugs as well as systemic corticosteroids and antibiotics [43]. Finally, low serum sodium, high serum potassium and elevated serum thyroid-stimulating hormone (TSH) may be detected before the diagnosis of AD [49].

An updated algorithm for the initial investigation of adrenal insufficiency has been recently published [50]. The first steps in the diagnostic process comprise random cortisol measurements, plasma renin and ACTH measurements, and a 250-µg synthetic ACTH stimulation test.

Therapy

The goals of GC replacement therapy in patients with AD is to abolish symptoms of GC deficiency, prevent adrenal crisis and, at the same time, not induce long-term side effects such as osteoporosis, obesity, hypertension and type 2 diabetes [29,30,51].

Currently, first-line therapy is hydrocortisone (15–25 mg/day) or cortisone acetate (25–37.5 mg/day) taken in divided doses twice or three times daily. A longer-acting GC, prednisolone (3–5 mg daily), can be used as a once or twice daily regimen in patients with poor compliance using multiple daily dose.
regimens [52]. Most patients with AD also need mineralocorticoid replacement, usually fludrocortisone (50–200 µg) once daily. The dose of fludrocortisone should be actively adjusted according to measurements of plasma renin, serum sodium and potassium, and blood pressure. Replacement of adrenal androgens in females has not shown consistent benefit [53].

New developments in the therapy of patients with AD include a dual-release hydrocortisone preparation (Plenadren®) and continuous subcutaneous hydrocortisone infusion (CSHI) using an insulin pump. These therapies have shown some advantages in patients with both diabetes and AD compared with conventional treatment: significant improvement in glycaemic control with dual-release hydrocortisone and more stable night-time glucose levels with CSHI [54,55].

It is essential to monitor for signs of under- and over-replacement with steroids during the follow-up of patients with AD. However, there is no reliable biomarker for this during GC replacement.

Adrenal Crisis

Patients with AD are at risk of developing adrenal crisis, a life-threatening condition if not rapidly treated with parenteral GCs and saline infusion. In a prospective study in patients with primary and secondary adrenal insufficiency, the incidence of adrenal crisis was 8.3 per 100 patient-years and adrenal crisis-associated mortality was approximately 6% [56]. Patients with AD are therefore taught to increase GC dose in the event of infection or other physical and mental stressful events in order to prevent a crisis. Continuous patient education plays a key role in self-management of AD and potential adrenal crisis [57]. In addition, a “steroid card” and medical alert patient identification tag are necessary to warn healthcare providers in the case of unconsciousness.

1.3 TYPE 1 DIABETES MELLITUS

Pathogenesis

T1DM results from destruction of pancreatic insulin-producing beta cells in the islets of Langerhans [58]. As in autoimmune AD, underlying genetic susceptibility is probably triggered by environmental agents, which leads to overt T1DM after a latent period. A number of different autoantigens have been described within beta cells but it is unclear which are involved in the initiation of the injury and which are secondary to injury. Autoimmune markers include islet-cell autoantibodies and autoantibodies to glutamic acid
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decarboxylase (GAD), insulin, tyrosine phosphatases IA-2 and IA-2b, and ZnT8.

**Epidemiology**

T1DM is one of the most common chronic diseases of childhood and its incidence and prevalence are increasing globally together with many other autoimmune diseases [59]. An estimated 30.3 million people of all ages had diabetes in 2015 in the USA, equivalent to 9.4% of the total population [60]. In Sweden, 44,466 adults and 7,203 children had T1DM in 2015 [61], which means that T1DM is approximately 40 times more common than AD.

Despite the considerable advances in diabetes care, T1DM is still associated with premature death [62]. A nationwide Swedish study showed a more than 2-fold higher risk of death (mainly from cardiovascular diseases and diabetes-related causes) in patients with on-target glycaemic control and an 8-fold higher risk of death in patients with poor glycaemic control compared with the general population [63]. In T1DM, nephropathy is a serious comorbidity increasing the risk of premature death and the most common diabetic complications are microvascular, especially in patients with long-standing diabetes [63,64]. A longitudinal US study within a single county explored patterns of cause-specific mortality in patients with T1DM [65]. Within the first 10 years after diagnosis, the leading cause of death was acute diabetic complications (74%) while, during the next 10 years, deaths were attributed to acute diabetic complications (15%), cardiovascular (22%), renal (20%) or infectious (18%) causes. After 20 years, chronic diabetic complications (cardiovascular, renal or infectious) accounted for more than 70% of all deaths, with cardiovascular disease as the leading cause of death (40%).

**Diagnosis**

T1DM usually presents with polyuria, polydipsia, weight loss and diabetic ketoacidosis (an acute, major, life-threatening complication). Diagnosis is based on the detection of abnormalities in glucose metabolism, including elevated fasting plasma glucose, elevated plasma glucose after an oral glucose tolerance test, and elevated HbA1c.

**Therapy**

Patients with T1DM are treated with insulin replacement. Description of the different insulin regimens, glycaemic control targets, self-management aspects and diabetes education is outside the purpose of this thesis. Nevertheless, it is worth mentioning some of the advances in T1DM care which have led to more
individualised management. These include patient education programmes, telecare intervention, routine assessment of comorbidities including their primary prevention, new insulin analogues, and continuous glucose monitoring.

Finally, HbA1c (a measure of glycosylation of haemoglobin) is an excellent diagnostic and therapeutic biomarker for patients with T1DM. By measuring HbA1c, which is a risk marker for future complications [63], physicians are able to get an overall picture of average blood glucose levels over a period of up to 12 weeks and tailor treatment accordingly.

1.4 TYPE 1 DIABETES & ADDISON’S DISEASE

Autoimmune Polyendocrine Syndrome Type 2

A patient with one autoimmune disorder is at increased risk of developing another autoimmune disease and the frequency of additional autoimmune diseases in T1DM shows an age-dependent increase [66]. Up to 60% of patients with autoimmune AD have an additional autoimmune condition (e.g. thyroid disease, T1DM) constituting APS2 [35-37]. Patients with autoimmune thyroid disease have an at least 10-times higher risk of developing other autoimmune diseases, e.g. pernicious anaemia, systemic lupus erythematosus, AD, coeliac disease [67].

Incidence and Prevalence

Having both T1DM and AD is rare, which may explain the paucity of outcome data in this patient group. Previously published data on the prevalence of concurrent T1DM and AD were the result of subanalyses in AD or T1DM populations. Between 10.7% and 12% of patients with AD also had T1DM [36,68]. The estimated prevalence of concurrent AD and T1DM in Western Norway was 20 per million [35] (Table 1). In a US population, 0.3% of patients with T1DM were found to have concomitant AD [66]. The risk for T1DM patients developing AD has not been determined. In addition, it is unknown whether women are more frequently affected than men by both T1DM and AD, and whether age at diagnosis of concurrent T1DM and AD peaks between 30 and 50 years of age as with AD alone.

Insulin vs Cortisol

Having both T1DM and AD is a complicated condition due to the counter-balancing effects of insulin and cortisol on glucose metabolism. The management of both diagnoses during an infection or other stressful events is
challenging in order to prevent adrenal crisis and manage hypoglycaemia or ketoacidosis. Because of these counter-balancing effects on glucose metabolism, patients with T1DM present with reduced insulin requirement when they develop severe GC deficiency due to AD [69]. In addition, a study of ten patients with both T1DM and AD showed different basal and meal-related insulin requirements compared with patients having only T1DM [70]. Apart from altered insulin requirements, data on prodromal signs and early indicators are missing in patients with T1DM that develop AD.

**Mortality**

With respect to prognosis, previous studies have shown an increased risk of potentially life-threatening adrenal crises among patients with both T1DM and AD versus patients with AD alone or the general population [56,71,72]. Another study has shown that patients with AD who also had diabetes had an increased risk of death compared to patients with AD alone [21]. Nevertheless, there is no previous national study on the mortality of patients with the combination of T1DM and AD.

**Other Knowledge Gaps**

At present, there is no consensus whether to perform regular screening for AD among patients with T1DM as the magnitude of risk is poorly known [73-77]. Finally, the complete lack of a biomarker in order to tailor GC replacement and balance this with insulin treatment (which is tailored by monitoring HbA1c) is profound.
2 AIMS

The hypotheses that generated the studies of this thesis were:

- *Autoimmune* AD is more common among patients with T1DM than in the general population;

- Patients with T1DM that develop AD may have specific prodromal signs that can help in predicting AD;

- Patients with T1DM, after they develop AD, have an additional increased risk of death;

- There are circulating biomarkers that reflect the biological action of GCs.

Consequently, the overall aim of this thesis was to study the epidemiology of the combination of T1DM and AD.

The specific aims were:

- To study the incidence and the relative risk of developing AD in patients with or without T1DM (Paper I).

- To study whether there are signs that denote the development of AD in patients with both T1DM and AD before they are diagnosed with AD (early indicators) (Paper II).

- To study overall mortality in patients with T1DM and AD (Paper III).

- To study putative biomarkers of GC action (Paper IV).
3 PATIENTS AND METHODS

3.1 EPIDEMIOLOGIC STUDIES

Study Design and Registries

With respect to the epidemiology of the combination of T1DM and AD, we conducted three nationwide, matched, observational cohort studies that merged data from six different Swedish National Registries (interlinked by a unique personal identification code) held by the Swedish National Board of Health and Welfare (Figure 1):

- National Diabetes Register (NDR): coverage >97%, data since 1998 [78];
- Swedish Inpatient Register (SNIR): complete coverage, data since 1987 [79,80];
- Cancer Register: complete coverage, data since 1958;
- Cause of Death Register: complete coverage, data since 1961;
- Longitudinal Integration Database for Health Insurance and Labor Market Studies (LISA; information on education level, income, civil status and county): complete coverage, data since 1990 [81];
- Prescription Drug Register: complete coverage, data since July 2005 [82].

*Figure 1. Overlap between the registries included in the epidemiological studies.*
All studies were approved by the Regional Ethical Review Board in Gothenburg, Sweden (diary numbers 563-12 and 776-14). All research complied with the Declaration of Helsinki.

**Study Subjects**

Subjects (and their comorbidities) were identified using the codes of the International Classification of Diseases, Ninth Revision (ICD-9) for years 1987–1996 and of ICD-10 for years 1997–2015. In the SNIR, the codes used to identify patients with AD were ICD-9 code 255.4 and ICD-10 codes E27.1 and/or E27.2. These search criteria for AD have previously shown high validity [21]. Patients with endogenous Cushing's syndrome and/or any disorders of the pituitary gland were excluded. For the identification of T1DM in the NDR, we used the diagnosis attributed to the patients by the physicians who reported them to the register. Details on all ICD-9 and ICD-10 codes used in the studies are presented in a supplementary table at the end of this thesis. Codes from ICD-7 were used for cancer diagnosis in the Swedish Cancer Register.

**Procedures**

We conducted three different studies. Table 2 shows the different study designs, study periods, groups of cases vs control subjects, the included registries, and the primary (AD or overall mortality) and secondary outcomes studied in each study. All patients with an AD diagnosis preceding the T1DM diagnosis were excluded in all studies. All patients deceased before the end of the observation period were excluded from the incidence and early indicators studies. Baseline (time point of inclusion in the study) for all studies was defined as the date of first registration of cases in the NDR.

**Statistical Methods**

At baseline, cases were compared with control subjects by descriptive statistics. For continuous variables with normal distribution, we performed two-sided independent samples t-test where p-value <0.05 was considered to be statistically significant. The difference between group proportions for binary variables was estimated by the difference in proportions along with the corresponding 95% confidence interval (CI). Table 2 shows the statistical methods and matching criteria used in each study.
### Table 2. Overview of the methodology in papers I-III.

<table>
<thead>
<tr>
<th></th>
<th>Paper I Incidence</th>
<th>Paper II Early indicators</th>
<th>Paper III Mortality</th>
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</thead>
<tbody>
<tr>
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<td>Cohort, 3 periods</td>
<td>Cohort, longitudinal</td>
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<td>T1DM who developed AD</td>
<td>T1DM who developed AD</td>
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<tr>
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<td>36,514</td>
<td>66</td>
<td>148</td>
</tr>
<tr>
<td>- No.</td>
<td>No T1DM</td>
<td>T1DM (no AD)</td>
<td>T1DM (no AD)</td>
</tr>
<tr>
<td>- No.</td>
<td>182,570</td>
<td>330</td>
<td>739</td>
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<tr>
<td><strong>Control subjects</strong></td>
<td>(5:1) age, sex, calendar year at inclusion in study, and county</td>
<td>(5:1) age, sex, duration of T1DM, and calendar year at inclusion in study</td>
<td>(5:1) sex, year of birth, time from diagnosis of T1DM to AD diagnosis for cases or T1DM duration until inclusion for controls, and calendar year of T1DM and AD diagnosis for cases or year of registration in the NDR for controls</td>
</tr>
<tr>
<td><strong>Matching criteria</strong></td>
<td>(5:1) age, sex, calendar year at inclusion in study, and county</td>
<td>(5:1) age, sex, duration of T1DM, and calendar year at inclusion in study</td>
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<td><strong>Outcomes studied</strong></td>
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<td>- primary</td>
<td>Relative risk of AD</td>
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<td>- secondary</td>
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<td>Cause of Death Register</td>
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<td>Analysis of covariance (ANCOVA), estimated group proportions, paired t-test</td>
<td>Standardised mean difference (SMD), Cox regression, Kaplan-Meier survival curves</td>
</tr>
</tbody>
</table>
Methodological Considerations

The strengths of all the studies were the reliability of the registries included and the diagnosis codes studied as well as the selected study procedures, including matching. More specifically, all registries have a high national coverage and are continuously validated. NDR provides detailed clinical data. Both the search criteria for AD in the SNIR and the reported main causes of death have been previously shown to have high validity [21,83]. Population-based national real-world data from the six different registries led to a panoramic approach and matching minimised the problem of confounding. By using control subjects also registered in the NDR, we reduced the problem of detection bias, as patients in this register have a similar frequency of clinical assessments.

Most studies are a compromise, where a hypothesis is studied in an available subgroup of a population and with restricted access to data due to different reasons. In this respect, our studies also had some methodological limitations. One of them was that there were no detailed clinical data for all matched control subjects without T1DM as they were not registered in the NDR.

In the incidence study, the hypothesis was that autoimmune AD is more common in patients with T1DM. Due to the lack of data on whether AD was autoimmune or not, we assumed that around 85% of the cases studied (as published previously in developed countries) actually had autoimmune AD.

In the early indicators study, a limitation was the limited access to drug prescription data, as no data were available for gastrointestinal, anti-anaemic and obstructive airway disease drugs for comparison with previous observations in patients with autoimmune AD. Moreover, we assessed the date of the first registration of the AD diagnosis as the time of the overt AD, when it is known that there is variation between the time of actual overt disease onset and its diagnosis.

In the mortality study, we studied the additional mortality in patients with T1DM after they were diagnosed with AD; however, we also included patients during data collection who were registered with both diseases for first time simultaneously. There were relatively few such patients (28 out of 148) and, taking in account the natural history of the two diseases, it is possible that the majority of them actually had T1DM first and then AD.
3.2 EXPERIMENTAL STUDY

Study Design

This was a single-centre, single-blind, randomised, two-period crossover, treatment clinical trial under the acronym BIOCORT (NCT02152553). The study was approved by the Ethics Review Board of the University of Gothenburg, Sweden (diary number 374-13) and written informed consent was obtained from all patients before participation.

Study Subjects and Treatment

Men or women with verified AD receiving stable and near-physiological GC doses and having no other comorbidities were eligible for the study.

Hydrocortisone intravenous infusion (1 mg hydrocortisone per 50 ml saline 0.9%) was adjusted in accordance with previous observations in healthy males [3,84] and interventions in both sexes [85,86]. The aim was to achieve a near-physiological circadian GC exposure with early morning increase in serum cortisol that would peak at 7 AM and trough concentrations at midnight (Figure 2A). Saline 0.9% infusion alone was administered to prevent adrenal crisis in the GC withdrawal period.

Interventions

Subjects were randomised before the first intervention to receive either intravenous hydrocortisone infusion in 0.9% saline or saline 0.9% infusion alone in a single-blind manner at least 2 weeks apart (Figure 2A). The day before each intervention, subjects were told not to take their mineralocorticoids and to take their usual dose of hydrocortisone. All subjects were admitted after an overnight fast and were discharged at 12 AM the day after. During each intervention, subjects received standard meals at fixed times and remained under minimal stress. Their consumption of coffee or tea was recorded to ensure consumption of the same amount and at same times during both interventions.

During each intervention, blood pressure, body temperature and body weight were measured. Serum and plasma were collected immediately before the start of intervention, at midnight and in the morning (of the second intervention day). Overnight urine was also collected.
Figure 2. Flow chart of the clinical and analytical part of the biomarker study. (A) Subjects with AD were studied twice over 26 h during physiological GC exposure (HC intervention) and GC withdrawal (saline intervention) at least 2 weeks apart. Serum and plasma samples were collected at midnight (15 h after start) and the next morning (22 h after start). (B) Transcriptomics were analysed from morning PBMC samples, whereas miRomics and metabolomics were analysed from both midnight and morning blood samples. (C) Stepwise integration of the ‘omic data into a network model.

Analyses (Including Bioinformatics)

The primary endpoint was defined as a network integrating changes in gene expression in PBMCs, miRNAs in plasma, and metabolites in serum between a state of near-physiological GC exposure (hydrocortisone intervention) and GC withdrawal (saline intervention) (Figure 2B).

The analyses performed in the study were:

- Plasma cortisol and cortisone analysed using liquid chromatography-mass spectrometry (LC-MS),
Addison’s Disease and Type 1 Diabetes Mellitus

- Urinary free GCs analysed using gas chromatography-mass spectrometry (GC-MS),
- Microarray gene expression analysis in peripheral blood mononuclear cells (PBMCs),
- MicroRNA (miRNA) analysis in plasma,
- Metabolic profiling of serum using both GC-MS and LC-MS.

The bioinformatics comprised data analysis of differential gene expression, Gene Ontology (to define biological pathways), gene expression regulated by miRNA, Causal Network Analysis (to assess regulatory relationships in the ‘omic data), network model construction and comparison (Figure 2C), and Similarity Network Fusion (to define similar groups of patients based on their combined ‘omic data).

**Statistical Methods**

For normally distributed quantitative variables, we performed paired samples t-test. For non-normally distributed quantitative variables, Wilcoxon rank test was performed. All statistical tests were two-sided and a p-value <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS program version 24 software for Mac.

Unsupervised analysis of transcriptomic and metabolomic data to assess how GC exposure grouped the study subjects was performed using Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) using the MixOmics plugin [87] for R. This is a statistical modelling tool that is robust to collinearity between the variables and provides insights into separation between experimental groups.

**Methodological Considerations**

In order to better understand the mechanistic pathways of GCs and to identify potential biomarkers of GC action in humans, a clinical study was found to be most appropriate. Most cell lines cannot survive without the presence of GCs and cannot be studied in a GC withdrawal state. Cell culture models using continuous GC treatment throughout the time course of the experiment are not representative of the physiology of GC exposure in vivo. Moreover, most animal models have different GCs compared with humans and their impact on circadian rhythm is also different. AD is a rare disorder but a unique experimental model due to the absence of adrenal GC production. A study using oral pharmacological GC exposure in AD patients or in patients with endogenous overt Cushing’s syndrome may add complexity to the model as
there are secondary effects of high GC exposure, such as activation of the mineralocorticoid receptor, impairment in glucose metabolism and increase in blood pressure.

The main strengths of this biomarker discovery study are the use of a human GC “knock-out” model (subjects with AD), the study design itself (randomisation, blinding, crossover), and the network analysis integrating data from three different functional levels (transcriptome, miRNA and metabolome). Moreover, interventions including meals and consumption of cafffeinated drinks were standardised, co-interventions were avoided, and subjects remained under minimal stress.

On the other hand, no power calculation was done because of the complexity and the “pilot” nature of the study. Power calculations are rather difficult in the context of ‘omic analysis as there may be variable effect sizes over many different ‘omic elements. Nevertheless, integration of multiple ‘omic layers allows reduction of background noise and allows us to increase confidence in the findings. The study design did not allow us to analyse any dose-response relationship or individual responsiveness (pharmacogenomics). Moreover, the gene expression analysis was performed in a single, important target tissue (PBMCs) for GCs and it is therefore possible that other results might be obtained if another tissue was studied. However, the measurement of miRNA and metabolites from peripheral blood (plasma and serum, respectively) at the same time, probably reflects the global effects of GCs.
### RESULTS

#### 4.1 EPIDEMIOLOGIC STUDIES

**Incidence and Relative Risk of AD among Patients with T1DM**

Between 1998 and 2013, 66 patients were diagnosed with AD among 36,514 patients with T1DM and 32 were diagnosed with AD among 182,570 matched control subjects without T1DM (general population). Baseline (first registration in the NDR for cases) demographics and clinical characteristics for the four different groups are presented in Table 3. The difference in mean age when AD was diagnosed between patients with T1DM or without T1DM was 6.3 years (95% CI: 0.4–12.2; p=0.036). Time to AD diagnosis in patients with or without T1DM is shown in Figure 3.

Table 3. Baseline (first registration in the NDR for cases) demographics and clinical characteristics among patients with or without T1DM and their subgroups with or without AD. Results are presented as n (%) for categorical variables and mean (SD) for continuous variables.

<table>
<thead>
<tr>
<th></th>
<th>T1DM (n=36,448)</th>
<th>T1DM+AD (n=66)</th>
<th>Controls (n=182,538)</th>
<th>Controls+AD (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at T1DM diagnosis (y)</td>
<td>15.3 (7.7)</td>
<td>13.6 (7.6)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Age at AD diagnosis (y)</td>
<td>35.2 (14.6)</td>
<td>36.4 (13.0)*</td>
<td>35.2 (14.6)</td>
<td>42.7 (15.2)*</td>
</tr>
<tr>
<td>Male</td>
<td>19,957 (54.8%)</td>
<td>39 (59.1%)</td>
<td>99,966 (54.8%)</td>
<td>14 (43.8%)</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>66 (16)</td>
<td>68 (15)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.2 (1.5)</td>
<td>8.4 (1.4)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*The difference between these groups was 6.3 years (95% CI: 0.4–12.2; p=0.036) (t-test).

The incidence of AD among patients with T1DM was 193 (95% CI: 152–245) per million patient-years compared with 18 (95% CI: 13–26) per million patient-years among matched control subjects without T1DM. The adjusted relative risk increase (hazard ratio, adjusted for age and sex) of developing AD in patients with T1DM was **10.8 (95% CI: 7.1–16.5) times** compared with control subjects without T1DM.
Early Indicators of AD in Patients with T1DM

Prior to baseline (first registration in the NDR)
The 66 cases with T1DM and AD from the incidence study were matched to 330 control subjects with only T1DM. Prior to baseline, prescription of thyroid and/or antithyroid drugs was higher in cases than in control subjects (9.1% vs 1.8%, Figure 4). There was no difference in adjusted mean HbA1c nor in the frequency of studied comorbidities between the groups.

Prior to AD diagnosis
Cases had the same adjusted mean HbA1c as their matched control subjects and five variables showed a higher frequency (statistically significant) in cases compared with control subjects:
- Infections requiring hospital admission (16.7% vs 2.1%),
- Multiple diabetic complications (13.6% vs 4.8%),
- Diabetic retinopathy (12.1% vs 2.1%),
- Prescription of thyroid and/or antithyroid drugs (28.8% vs 7.0%),
- Prescription of glucagon (18.2% vs 6.4%).

The 66 cases with T1DM who developed AD were then followed for up to 2 years after the AD diagnosis. Mean HbA1c was at the same level before and after AD diagnosis. The frequency of diabetic complications, autoimmune diseases and other comorbidities also remained at the same level. Adrenal
crisis was recorded in two patients within 2 years after AD diagnosis, while none were observed before AD diagnosis. A trend for higher prescription of antihypertensive drugs was observed in T1DM and AD patients after AD diagnosis compared with the period before diagnosis (47.0% vs 30.3%; difference 16.7% [95% CI: –4.2% to 37.5%]). Drug prescription data were available after AD diagnosis in up to 56 of 66 cases with T1DM and AD. Among the 66 cases, 78.8% were treated with hydrocortisone, 4.5% with prednisolone and 3.0% with cortisone acetate. Prescription data for fludrocortisone (mineralocorticoid) were available in 55 of the 66 cases, among which 57.6% were treated with fludrocortisone and 25.8% were not treated.

*Figure 4. Frequencies of prescription of thyroid/antithyroid drugs between patients with T1DM and AD and T1DM alone prior to baseline (inclusion to the study) and prior to AD diagnosis (within 2 years). The statistical significant difference in frequencies (corresponding 95% CIs) between the two groups and at both time points are presented.*

**Overall Mortality in Patients with T1DM and AD**

In the NDR and SNIR, we identified 148 patients (77 men, 71 women) with T1DM who then developed AD or were registered with both diagnoses (28 out of 148) at the same time between 1996 and 2012. These cases were matched 1:5 to 739 control subjects (384 men, 355 women) with T1DM based on sex, year of birth, time from diagnosis of T1DM to AD diagnosis for cases or T1DM duration until inclusion for control subjects, and calendar year of T1DM and AD diagnosis for cases or year of registration in the NDR for control subjects. At baseline, mean±SD HbA1c was 64±14 mmol/mol or 8±1.3% in cases and 63±15 mmol/mol or 7.9±1.4% in control subjects. The proportion of diabetic retinopathy and multiple diabetic complications were
clearly higher in cases than in controls (Table 4). Medical history of infections requiring hospital admission was markedly increased in the T1DM and AD group compared with the T1DM controls (SMD=46.35).

Table 4. Baseline (first registration in the NDR) clinical data in cases with T1DM and AD, and their matched control subjects with T1DM. Data presented as n (%). SMD is a measure of size effect, with imbalance between the groups defined as an absolute value >20%.

<table>
<thead>
<tr>
<th></th>
<th>T1DM+AD (n=148)</th>
<th>Controls with T1DM (n=739)</th>
<th>SMD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic nephropathy</td>
<td>8 (5.4%)</td>
<td>15 (2.0%)</td>
<td>–17.91</td>
</tr>
<tr>
<td>Diabetic retinopathy</td>
<td>31 (21.0%)</td>
<td>52 (7.0%)</td>
<td>–40.93</td>
</tr>
<tr>
<td>Diabetic neuropathy</td>
<td>11 (7.4%)</td>
<td>19 (2.6%)</td>
<td>–22.44</td>
</tr>
<tr>
<td>Diabetic angiopathy</td>
<td>8 (5.4%)</td>
<td>11 (1.5%)</td>
<td>–21.60</td>
</tr>
<tr>
<td>Multiple diabetic complications</td>
<td>21 (14.0%)</td>
<td>33 (4.5%)</td>
<td>–33.91</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>9 (6.1%)</td>
<td>75 (10%)</td>
<td>14.94</td>
</tr>
<tr>
<td>including acute myocardial infarction</td>
<td>11 (7.4%)</td>
<td>36 (4.9%)</td>
<td>–10.67</td>
</tr>
<tr>
<td>History of congestive heart failure</td>
<td>2 (1.4%)</td>
<td>10 (1.4%)</td>
<td>0.02</td>
</tr>
<tr>
<td>History of stroke</td>
<td>3 (2.0%)</td>
<td>11 (1.5%)</td>
<td>–4.10</td>
</tr>
<tr>
<td>History of infections requiring hospital admission</td>
<td>46 (31%)</td>
<td>92 (12%)</td>
<td>–46.35</td>
</tr>
<tr>
<td>History of cancer</td>
<td>2 (1.4%)</td>
<td>25 (3.4%)</td>
<td>13.39</td>
</tr>
</tbody>
</table>

The observed number of deaths was 29 in the 148 cases (20%) and 35 in the 739 controls (4.7%). The adjusted relative risk increase (hazard ratio adjusted for years at baseline, years at T1DM diagnosis, duration between them and sex) in overall mortality of the T1DM and AD group was **4.3 (95% CI: 2.6–7.0) times** compared with matched T1DM controls. The cumulative overall mortality in cases vs control subjects is shown in Figure 5.

Figure 5. Cumulative overall mortality in patients with T1DM and AD vs control subjects with T1DM.
Cause-specific Mortality in Patients with T1DM and AD

There were differences in the main causes of death between cases with T1DM and AD and matched control subjects with T1DM (Figure 6). The most common main cause of death was due to T1DM complications in both groups, whereas deaths from unknown cause and infections were more frequent among patients with T1DM and AD. Death from cancer (as main cause of death) was more frequent among control subjects with T1DM.

Figure 6. Main causes of death in patients with T1DM and AD vs control subjects with T1DM.
4.2 EXPERIMENTAL STUDY

Baseline Characteristics

Eleven subjects with AD with a median age of 50 (range, 25–57) years and a median disease duration of 23.5 (range, 1–33) years were included in the study. Ten (4 women, 3 of them post-menopausal) completed all aspects of the study. The median daily dose of hydrocortisone prior to the study was 30 (range, 20–30) mg and nine subjects received fludrocortisone at a median daily dose of 0.1 (range, 0.1–0.2) mg.

Serum and Urinary Glucocorticoids

There was no difference between the two interventions in serum cortisol or cortisone collected at baseline, while both were markedly higher in the morning of the second day during GC exposure (both p<0.001). Interestingly, both were detected in all subjects’ morning samples during GC withdrawal. However, both overnight urine cortisol and cortisone excretion were under the limit of detection during GC withdrawal, indicating very low GC exposure.

There was no difference in systolic and diastolic blood pressure, body weight, serum sodium and potassium, and plasma glucose concentrations between the two interventions both at baseline and in the morning of the second day (when samples were collected for ‘omic analyses).

Raw ‘Omic Data

Firstly, a transcriptomics analysis of PBMC samples from the morning of the second day was performed. By comparing the two interventions, 289 differentially expressed gene probe sets were identified (p<0.001, q<0.2) consisting of 234 unique known genes (data not shown).

In the same comparison, nine of 252 analysed plasma miRNAs (from the morning of the second-day samples) were shown to be differentially expressed between the two interventions (Wilcoxon signed rank test p<0.05, data not shown). The nine miRNAs were then predicted to interact with 46 of the 234 unique, differentially expressed genes from the transcriptomics analysis.

The transcriptomics and miRNA analyses were then followed by serum metabolomics analysis (both midnight and morning) using both GC-MS and LC-MS. The analysis of metabolomic data using OPLS-DA showed clear clustering of metabolites by intervention and time points (Figure 7). Comparison of 164 metabolite fragments (82 derived from GC-MS and 82
from LC-MS) during the two interventions revealed a clear distinction between GC exposure and GC withdrawal. A paired t-test analysis identified 21 metabolite fragments from GC-MS and 17 metabolite fragments from the LC-MS analysis that were significantly different ($p<0.05$) between the two interventions in the morning of the second-day samples (data not shown).

Figure 7. Separation between experimental groups by OPLS-DA analysis for GC-MS data. Black squares depict midnight and morning samples from the GC withdrawal intervention and red squares depict midnight and morning samples from the intervention with physiological GC exposure.

**Interactome Network Model and Transcriptome Grid Diagram (Step 1 in Network Analysis)**

Biological pathways associated with differential gene expression observed between the two interventions in PBMCs collected in the morning of the second day were assessed. A range of classically GC responsive pathways were present, including GC receptor signalling and NF-κB signalling, along with a range of metabolism-linked pathways. These pathways together imply GC activity during the GC exposure intervention.

We generated an interactome network model (with 2467 nodes) based on the 234 unique, known genes with differential expression between the two interventions in the morning of the second day. Hierarchy of network modules based on network centrality score, a measurement of the proximity of the module to the centre of the network, is known to be related to functional importance [88-90]. We therefore defined the modules in the network model of differential gene expression associated with GC exposure in order to facilitate the integration of the other ‘omic datasets (miRNAs and metabolites).
The differential gene expression within the central core (ten genes/proteins) of each of the top 25 network modules were visualised as a **transcriptome grid diagram** (in the middle of Figure 8) and was used as the basis for further analysis. The central genes of each network module were all shown to have relatively increased connectivity compared with the whole human interactome, and these observations suggest functional relevance and confirm network robustness.

A range of genes in the interactome network model associated with GC action were shown to have evidence of NR3C1 binding to associated regulatory DNA elements (FKBP5, ZBTB16, IGF1R, PER1, TSC22D3, and NCOR2). These data were derived from the ENCODE database using a range of cell lines in response to a dexamethasone dose-response.

**Integration of miRomic and Metabolomic Data (Steps 2 and 3 in Network Analysis)**

In the differentially expressed genes were then mapped, six of the nine genes differentially expressed miRNAs (in the morning of the second-day samples). Of these six miRNAs, five mapped to the central core of the transcriptome network module (Figure 8A).

Independently from the first mapping, the 21 differentially regulated metabolite fragments in the morning of the second-day samples from the GC-MS were mapped to the differentially expressed genes. This analysis demonstrated that some of these metabolites mapped to the central core of the transcriptome networks modules. It should be noted that this analysis showed the existence of two separate networks of metabolites (Figure 8B).

*In conclusion*, findings from steps 1–3 include differentially regulated miRNAs and metabolites, which are associated with the differential gene expression and GC exposure.
Figure 8. MiRomic data and metabolomic data mapped independently to the interactome network model of differential gene expression (visualised by the its grid diagram) between the two interventions in morning samples. (A) Each miRNA is mapped to the position of the gene in the transcriptomic network model that it regulates. (B) The analysis demonstrated two separate network models (encircled in violet or orange colour) generated by combining transcriptomic and metabolomic data.
5 DISCUSSION

Higher Risk of AD among Patients with T1DM

Our incidence study estimated the incidence of AD among patients with T1DM in Sweden at 193 per million patient-years. Patients who already had T1DM showed a more than 10-fold higher risk of developing AD, which appeared at a younger age than in patients without T1DM. Finally, there seemed to be a male predominance in patients with T1DM that developed AD, which is in line with the male predominance of T1DM [59] and in contrast to the female predominance of AD in the general population [35,40-42].

These findings suggest that healthcare professionals who follow patients with T1DM should be aware for the possibility of the development of concomitant AD as this is much more common in this patient group. In contrast to AD in the general population where there is a female predominance and age at diagnosis peaks at 30–50 years of age, AD is more common in men and at younger age in patients with T1DM.

Early Indicators of AD in Patients with T1DM

To our knowledge, this is the first time that prodromal or early signs of AD have been systematically studied in patients with T1DM. We found that the following were more frequent among patients with T1DM who subsequently developed AD:

- Treatment for thyroid diseases,
- Severe infections requiring hospital admission,
- Multiple diabetic complications and retinopathy in particular,
- Rescue therapy for hypoglycaemia.

In our study population, among patients with T1DM who developed AD, up to 33% had treatment with thyroid and/or antithyroid drugs before and after the diagnosis of AD (4-fold higher in cases after AD diagnosis than in controls with T1DM). This is in line with a previous Swedish study reporting that 38% of patients with AD also had T1DM and autoimmune thyroid disease [43]. Already, at more than 2 years before our study patients were diagnosed with AD, they had a higher frequency of treatment for thyroid disease versus controls with T1DM alone. Our study therefore suggests that there should be an increased alertness for the possibility of developing AD (APS2) in patients with concurrent T1DM and autoimmune thyroid disease.
The appearance of severe infections requiring hospital admission in patients with T1DM may also alert the physician for the possibility of AD development. In our study population there was a significant difference in the frequency of such infections prior to AD diagnosis compared with control subjects (17% vs 2%) and a similar trend was observed many years before the AD diagnosis (data not presented). Previous studies in patients with AD have shown a higher frequency of infections, including those requiring hospital admissions or a higher frequency of treatment with systemic antibiotics [43,91,92]. There are two possible explanations for this observation: either this is an effect of the increased vulnerability to infections in patients with AD as suggested in two previous studies [93,94] or that patients with concomitant T1DM have a lower threshold of being admitted to hospital because of dysregulated glycaemic control (or risk for that) during an infection. The latter explanation is less likely due to the case-control design of our study.

Before the diagnosis of AD, patients with T1DM who developed AD had a 6-fold higher frequency of diabetic retinopathy compared with matched control subjects. This finding might be related to the underlying systemic proinflammation or inflammation in patients with AD [95], as there is increasing evidence that diabetic retinopathy has an inflammatory background [96]. It is also shown that longer diabetes duration and poorer glycaemic and blood pressure control are strongly associated with diabetic retinopathy [97], but there was no difference in these parameters between cases and control subjects in the current study, strongly indicating that the development of AD per se is responsible for this increase.

Glucagon is a therapy for acute hypoglycaemia and its prescription may, in some individuals, denote higher risk (or actually higher incidence) of hypoglycaemia. In our study, close to their AD diagnosis, patients with T1DM were prescribed glucagon at a rate 3-times higher than their matched controls with T1DM, suggesting higher risk (or incidence) of severe hypoglycaemia.

These findings suggest that healthcare professionals who follow patients with T1DM should be alerted when such potential early clinical indicators arise and evaluate their patients for the risk of concomitant AD development. How this is best done in patients with T1DM is unclear. Morning serum cortisol and ACTH, and short synthetic ACTH stimulation test are generally the assessments of choice for the diagnosis of AD. Whether their specificity and sensitivity are as high in patients with T1DM has not been studied.

**Increased Mortality**

Our nationwide mortality study in patients with T1DM and AD showed a 4-fold increased risk of overall mortality versus matched controls with T1DM.
This risk is on the top of the already increased mortality in Swedish patients with T1DM [63]. The most common main cause of death in patients with T1DM and AD was death related to diabetic complications, whereas the national Swedish study in patients with AD has shown cardiovascular causes as the most common cause of death [21], and another national Swedish study in patients with T1DM has shown both cardiovascular and diabetes-related causes as the most common causes of death [63]. In our study, infections and unknown causes of death were more common in patients with T1DM and AD than in those with T1DM alone.

The increased rate of death from diabetes-related complications and infections might be due to their higher proportion already before the diagnosis of AD. In both our early indicators and mortality studies, where we followed up patients with T1DM who developed or did not develop AD (thus with different study periods and in partially overlapping populations), we observed a higher frequency of diabetic complications and infections requiring hospital admission before the diagnosis of AD versus matched controls with T1DM. The explanation for this is unclear, but it may be related to the fact that management of T1DM may be more difficult if the intrinsic regulation of cortisol secretion is impaired, which is likely to occur well in advance of the actual diagnosis of AD. Adrenal medulla function may also be impaired in patients with AD [98] and this may be deleterious in patients with T1DM when they are dependent on a catecholamine rescue mechanism during hypoglycaemia.

At inclusion in the mortality study, the degree of cardiovascular comorbidities, cancer and glycaemic control did not differ markedly between cases and matched control subjects with T1DM, suggesting an impact of AD on excess mortality. A possible explanation for this deleterious effect of AD on mortality rate might be the counter-balancing metabolic effects of insulin and cortisol, the main treatments for T1DM and AD, respectively.

Premature death due to cancer was more common among control subjects with T1DM versus those with T1DM and AD, suggesting that AD or its treatment with GCs may modify the increased risk of cancer death among patients with T1DM. A previous national Swedish study has shown increased cancer risk in patients with T1DM compared with the general population [99]. Hyperglycaemia, insulin resistance, hyperinsulinaemia and the effects of diabetes treatment have all been suggested as possible mechanisms for the increased cancer risk in type 2 diabetes [100]. Little is known about the effect of AD and its treatment on these parameters in patients having both T1DM and AD.
Risk of Adrenal Crisis

There were some interesting findings in our epidemiological studies concerning the risk of adrenal crisis in patients with the combination of T1DM and AD. Previous studies have shown an increased risk of potentially life-threatening adrenal crises among patients with both T1DM and AD versus those with AD alone or the general population [56,71,72]. Our mortality study supports these previous observations not only by identifying patients with adrenal crisis as the main cause of death but also by showing higher mortality from infections and unknown causes. These deaths may be explained by untreated or inadequately treated adrenal crises, severe hypoglycaemia or their combination.

As adrenal crisis can be prevented by an adequate rescue regimen, our studies indicate the high risk of premature death from stress-related events when patients with both T1DM and AD are not treated properly. Because of the complex metabolic interplay between insulin and cortisol in patients with T1DM and AD, targeted information and education are needed to manage both diagnoses during an infection or other stressful events.

Routine Screening of Patients with T1DM for AD

The risk of associated autoimmune diseases (most commonly Hashimoto’s thyroiditis, coeliac disease, Graves’ disease, rheumatoid arthritis and vitiligo) is well defined in patients with T1DM [66]. The latest recommendations (2018) from American Diabetes Association concerning assessment of comorbidities suggest the routine evaluation of TSH for thyroid-associated diseases in patients with T1DM but not for AD [77]. The recommendation to screen for thyroid diseases but not AD in T1DM patients may be related to the rarity of the combination of T1DM and AD, and the fact that TSH monitoring is less expensive, less laborious and easier to interpret. There are currently no guidelines for routine screening for AD among patients with T1DM (with or without autoimmune thyroid disease) [73-76].

In the incidence study, we demonstrated that AD is 10 times more common in patients with T1DM and, in the mortality study, that AD markedly increases the overall risk of death in patients with T1DM, which both advocate for AD screening. Potential laboratory assessments for that would be serum morning cortisol and ACTH, plasma renin or 21-hydroxylase antibodies as reported in previous studies [39,73,101,102]. Nevertheless, a circulating biomarker (in a similar manner to HbA1c for the assessment of suspected diabetes) could be a complementary, or possibly even a first-line, laboratory measurement in the future for AD screening.
Discovery of Novel Putative Biomarkers of GC Action

In our experimental study, we succeeded in generating two completely different states of GC exposure (near-physiological vs nearly undetectable) in subjects with AD, in whom we studied the short-time actions of GCs with the help of multiple ‘omic methods. The novelty of the study is that its results may improve the understanding of mechanistic pathways by which GCs act and identifying potential biomarkers from gene expression, metabolome and miRNAs derived from a step-wise integration of different ‘omic data into a network.

The concentrations of cortisone and cortisol in serum and urine from the interventions both at baseline and in the morning of the second day as well as the absence of major confounders in the form of secondary events related to the GC withdrawal state indicate the success of the model. Moreover, the similar vital parameters, and glucose and electrolyte levels reduce the possibility of increased adrenergic drive related to the GC withdrawal state. The detectable levels of serum cortisol throughout GC withdrawal may be explained by residual adrenal steroid secretion in some patients [103] or possible conversion of cortisone to cortisol in liver and adipose tissue [104].

Some of the transcriptomic data were in line with previous findings, which supports the validity of the study model. Comparison between the central genes of each network module in the transcriptome network model with those involved in circadian clock rhythms [105], steroidogenesis [106], GC responsiveness (mainly in malignancies) [107-110], and a recent published list of genes with elevated expression in the adrenal cortex [111] showed that many of these known genes are present in our network, such as PER1, CRY1, CRY2, and GAB1. In the transcriptomic network modules, TSC22D3, which encodes the anti-inflammatory GC-induced leucine zipper protein (GILZ), occupied central positions. Expression of this gene appears to play a key role in the anti-inflammatory and immunosuppressive effects of GCs.

Some of our findings in the metabolome analysis were also in line with a recently published metabolomic study in patients with congenital adrenal hyperplasia on long-term GC treatment [112]. In our study, metabolites such as palmitic acid, palmitoleic acid, docosahexaenoic acid, tryptophan and octanoylcarnitine revealed a clear distinction between GC exposure and GC withdrawal (data not presented). In contrast to the gene expression and metabolome data, all the miRNA findings comparing short-time GC exposure to withdrawal were novel.

In conclusion, the results obtained from this clinical model showed a coherence and were in alignment with what is known about GC response, indicating relevance in the clinical model.
6 CONCLUSION

The main findings of this thesis are summarised in Figure 9.

*Figure 9. Main thesis findings.*

This thesis has demonstrated a higher risk of AD among patients with T1DM and higher mortality in patients with concurrent T1DM and AD, **indicating the need for an improved strategy in the management of these patients.**

Patients with T1DM face an approximately 10.8-times increased risk of developing AD compared with the general population; furthermore, they develop AD approximately 6.3 years (mean value) earlier than those not having T1DM. Signs, such as medical treatment for thyroid disease, infections requiring hospitalization, prescription of glucagon and diabetic retinopathy, in patients with T1DM may raise the suspicion of undiagnosed concomitant AD. Also, when patients with T1DM are diagnosed with AD, they face an additional risk for premature death that is approximately 4.3-times higher than those with T1DM alone.

Concerning improvement in the management of these patients with T1DM and AD, we need a biomarker to individualise GC replacement therapy in a similar manner to HbA1c, being an excellent biomarker for insulin replacement therapy. The novel findings arising from our experimental study indicate such potential biomarkers for further validation.
7 FUTURE PERSPECTIVES

Our data, which identified for the first time the impact of AD on outcome in patients with T1DM, support a low threshold for biochemical evaluation of AD development among patients with T1DM. In our opinion, this supports the re-evaluation of a screening strategy for AD in patients with T1DM during their follow-up. At present, there is no national (in Sweden) or international consensus concerning this kind of screening.

The combination of T1DM and AD is rare, and more uncommon than other combinations of autoimmune diseases in T1DM patients. Nevertheless, data on the deleterious effect of AD (when it is diagnosed) on mortality rate and the increased risk for adrenal crises versus patients with AD alone support the need for an improved management strategy of these patients.

For patients already diagnosed with this combination, who struggle to balance the risks of hypoglycaemia and adrenal crisis, which both potentially lead to coma, targeted information and education are needed. A possible way of achieving this might be by patient education programmes similar to those that have been developed for patients with T1DM or AD. Another way, both for patients and healthcare providers, might be by distribution and completion of special “insulin-steroid cards” similar to those that have already been implemented for patients with AD alone.

One possible next step in the understanding of the metabolic interplay between insulin and cortisol in these patients might be an interventional study with continuous measurement of glucose and GC levels in patients with different combinations of various insulin and GC preparations. Such a study might provide more insights into the mechanisms behind this metabolic interplay and might answer the question of which is the best combination of replacement therapy with different insulin and GC preparations to mitigate the risk of premature death.

Moreover, in order to come closer to a valuable and clinically useful biomarker(s) of GC action, we have to validate and confirm our findings (gene expression, miRNAs and metabolites) in other populations (e.g. in patients with GC over-production/over-substitution and healthy individuals) or different states of GC substitution in patients with AD (with or without T1DM).

Such a circulating biomarker (in a similar manner to HbA1c in diabetes assessment) might become a complementary, or even first-line, laboratory measurement for routine screening for AD in patients with T1DM in the future. Moreover, it might also useful for individualising GC treatment in patients with AD, exactly as HbA1c does in T1DM.
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My mother Andriana, my father Vasilis, my sister Vivi, my grandparents Katerina and Konstantinos.

My wife Eva and our son Vasilis.
REFERENCES


## APPENDIX

*Supplementary table: ICD-9 and ICD-10 codes of the diagnoses used in the studies*

<table>
<thead>
<tr>
<th>Group of diagnoses</th>
<th>Diagnosis</th>
<th>ICD-9 code</th>
<th>ICD-10 code</th>
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<tbody>
<tr>
<td>Addison's disease</td>
<td>Addison's disease</td>
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<td>Addisonian crisis</td>
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<tr>
<td>Diabetes mellitus</td>
<td>All types</td>
<td>250</td>
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</tr>
<tr>
<td></td>
<td>Type 1</td>
<td>–</td>
<td>E10</td>
</tr>
<tr>
<td></td>
<td>Type 2</td>
<td>–</td>
<td>E11</td>
</tr>
<tr>
<td></td>
<td>Malnutrition-related</td>
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<td>E12</td>
</tr>
<tr>
<td></td>
<td>Other specified</td>
<td>–</td>
<td>E13</td>
</tr>
<tr>
<td></td>
<td>Unspecified</td>
<td>–</td>
<td>E14</td>
</tr>
<tr>
<td>Cushing’s syndrome (drug-induced Cushing syndrome not included)</td>
<td></td>
<td>255.0</td>
<td>E24.0–E24.1, E24.3–E24.9</td>
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<tr>
<td>Hypofunction and other disorders of the pituitary gland</td>
<td>Disorders of the pituitary gland and its hypothalamic control</td>
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<td>–</td>
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<tr>
<td></td>
<td>Pituitary neoplasia</td>
<td>227.3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Hypofunction and other disorders of the pituitary gland</td>
<td>–</td>
<td>E23</td>
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<tr>
<td>Malignant neoplasm of the adrenal gland</td>
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<td>194.0</td>
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<td>Anomalies of the adrenal gland (congenital)</td>
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<td>Post-procedural adrenocortical (-medullary) hypofunction</td>
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<td>–</td>
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<td>C00–C97</td>
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<tr>
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<td>410</td>
<td>I21</td>
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<tr>
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<td>410–414</td>
<td>I20–I25</td>
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<tr>
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<td>427.3</td>
<td>I48</td>
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<td>Cardiac dysrhythmias (atrial fibrillation included)</td>
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<td>427</td>
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<td>428</td>
<td>I50</td>
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<td>I10–I15</td>
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<td>714.0–714.2, 714.8, 714.9</td>
<td>M05, M06</td>
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<td>710.0</td>
<td>M32</td>
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<tr>
<td>Stroke</td>
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<td>431, 432, 434</td>
<td>I61–I64</td>
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<tr>
<td>Haemorrhagic stoke</td>
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<td>431, 432</td>
<td>I61, I62</td>
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<tr>
<td>Cerebral Infarction</td>
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<td>434</td>
<td>I63</td>
</tr>
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<td>Diabetes mellitus-related complications</td>
<td>Ophthalmic</td>
<td>250.5</td>
<td>E10.3, E11.3, E12.3, E13.3, E14.3</td>
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<td>-----------------------------------------</td>
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<tr>
<td>Neurological</td>
<td>250.6, 357.2</td>
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<tr>
<td>Peripheral circulatory</td>
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<td>E10.5, E11.5, E12.5, E13.5, E14.5, I79.2</td>
<td></td>
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<tr>
<td>Other specified</td>
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<td>Unspecified</td>
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<tr>
<td>DM with foot ulcer</td>
<td>–</td>
<td>E10.6D, E11.6D</td>
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<tr>
<td>Amputation of limb</td>
<td>E878.5</td>
<td>Y83.5</td>
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<table>
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<th>Infections requiring admission to hospital (inclusive erysipelas)</th>
<th>Infections</th>
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<th>A00–A49, J09–J18, J20–J22, N39.0</th>
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<td>Erysipelas</td>
<td>035</td>
<td>A46</td>
<td></td>
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</tbody>
</table>

| Diabetic hypoglycaemia | 250.8 | E.16.0, E16.2, E10.6A, E11.6A |

| Chronic kidney disease | 585, 586 | N18, N19 |

ICD-9: International Classification of Diseases, Ninth Revision; ICD-10: International Classification of Diseases, Tenth Revision