Clues to Diagnosis and Clinical Outcomes in Autoimmune Addison's Disease

Åse Bjorvatn Sævik

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2023

UNIVERSITY OF BERGEN

Clues to Diagnosis and Clinical Outcomes in Autoimmune Addison's Disease

Åse Bjorvatn Sævik

Thesis for the degree of Philosophiae Doctor (PhD)
at the University of Bergen

Date of defense: 23.10.2023

Year: 2023

Title: Clues to Diagnosis and Clinical Outcomes in Autoimmune Addison's Disease

Name: Åse Bjorvatn Sævik

Print: Skipnes Kommunikasjon / University of Bergen

Where then does wisdom come from? Where does understanding dwell?
God understands the way to it, and He alone knows where it dwells.
When He established the force of the wind and measured out the waters,
When He made a decree for the rain and a path for the thunderstorm,
Then He looked at wisdom and appraised it; He confirmed it and tested it.
The Bible (Job 28 verses 20, 23, 25-27)

Scientific Environment

This work was conducted at the Department of Clinical Science, Faculty of Medicine, University of Bergen. Marianne Øksnes, Ph.D., has been the main supervisor, and Professor Eystein Husebye and Sophie Bensing, Ph.D., have been co-supervisors.

Key collaborators have included the Norwegian Registry for Organ-specific Autoimmune Diseases (ROAS), the Department of Internal Medicine at Haukeland University Hospital, and research partners throughout Norway (**Papers I-III**), Sweden (**Papers I-III**), and Germany (**Papers II-III**). Financial support has been provided by the University of Bergen, the Western Norway Health Authorities, the K.G. Jebsen Foundation, the Research Council of Norway, the Novo Nordisk Foundation, the Internal Medicine Association of Norway, and the Legate of Dr. Nils Henrichsen and Wife Anna Henrichsen.

Acknowledgments

To all who have accompanied me on this journey: thank you.

To my main supervisor Marianne Øksnes; I am forever thankful for your invaluable guidance and earnest care, and for patiently sharing your outstanding clinical and scientific expertise. Likewise, co-supervisor Eystein Husebye, a never-ending thank you for introducing me to the captivating world of adrenal endocrinology and for always believing in me and cheering me on. I am indebted to you, co-supervisor Sophie Bensing, for your extensive contributions to the planning and conduction of the studies.

A great thanks to my "partner in crime", Anna-Karin Åkerman, and the best office mate, Elinor Vogt, for valuable discussions and shared laughs and sighs. My heartfelt gratitude extends to all my colleagues in the Husebye research group as well as everyone at the Endocrinology Department at Haukeland for a welcoming and outstanding work environment. Especially thanks to the heads of the Endocrinology Department, Hrankell Thordarson, Martina Moter Erichsen, and Kristian Løvås, for graciously providing me with facilities to perform the clinical study. Mona Eliassen and Nina Jensen; thank you for your invaluable assistance in organizing patient visits, and to Elisabeth Tombra Halvorsen, for the best handling of patient blood samples. Paal Methlie; without your capable mind and many sacrificed nights of sleep, the residual function study would never have been possible. A great thank you to Lars Ertesvåg Breivik: you seem to know the solution to any practical issue I encounter. To all co-authors: thank you for the wonderful collaboration and all your hard work. Thank you to the Departments of Research, e-Health, and Technology and Innovation at Sørlandet Hospital, for a heartwarming welcome and generous coffee supply. A special thanks to the entire team behind *Endopodden* for making the dream of an educational podcast in endocrinology come true.

I wish to express my gratitude to all study participants. Thank you for generously lending your time and sharing your experiences with me. You are the best teachers.

I am forever thankful to my dearest parents, grandparents, and family-in-law, for your invaluable support and prayers, and to Signe and Kristine, for keeping your older sister relatively young.

My greatest thanks go to the love of my life and my best friend, Fredrik, for your selfless devotion, kindness, and support, and for patiently enduring euphoric monologues on everything from the awe-inspiring adrenal glands to Oxford commas. You are simply the best, and I am immensely fortunate to be your wife. Also, a never-ending thank you to our beloved sons, Håkon and Øyvind, for all the wonder and joy you bring by simply being - and for (at times even patiently) accompanying me at numerous meetings and writing sessions.

Abbreviations

21OH:	21-hydroxylase
21OHab:	21-hydroxylase autoantibodies
ACTH:	Adrenocorticotropic hormone
$ACTH_{1-24}$:	Synthetic ACTH
AAD:	Autoimmune Addison's disease
AddiQoL-30:	Addison-specific quality of life 30-item
AIRE:	Autoimmune regulator gene
APS1, 2:	Autoimmune Polyendocrine Syndrome type 1, type 2
BMI:	Body mass index
CBG:	Cortisol binding globulin
CD4:	T-cell surface glycoprotein
CRH:	Corticotropin-releasing hormone
CRP:	C-reactive protein
CVD:	Cardiovascular disease
DHEA(S):	Dehydroepiandrosterone (sulfate)
FDR:	False discovery rate
FGF21:	Fibroblast growth factor 21
GC(s):	Glucocorticoid(s)
HRQoL:	Health-Related Quality of Life

IL6:	Interleukin 6
LC-MS/MS:	Liquid chromatography tandem mass spectrometry
MC(s):	Mineralocorticoid(s)
MC1-5R:	Melanocortin receptor 1-5
MCP1:	Monocyte chemoattractant protein 1
NPX:	Normalized protein expression
PAI:	Primary adrenal insufficiency
PDL2:	Programmed cell death ligand 2
POMC:	Pro-opiomelanocortin
RAGE:	Receptor for advanced glycation end products
RAND-36:	Research and development 36-Item
RAF:	Residual adrenocortical function
ROAS:	Registry for Organ-specific Autoimmune Diseases
TPO:	Thyroid peroxidase
TSH:	Thyroid-stimulation hormone
Z.:	Zona

Abstract

Delayed diagnosis of autoimmune Addison's disease (AAD) is common and increases the risk of a life-threatening adrenal crisis. In established AAD, there seem to be inter-patient differences in risk for adrenal crisis, cardiovascular disease (CVD), and health-related quality of life (HRQoL). Blame is typically put on excess and unphysiological glucocorticoid replacement, but which patients may be more prone to worse outcomes and why is not fully understood. This thesis has aimed to explore clues to early diagnosis and the variation in clinical outcomes in AAD.

A retrospective audit of routine laboratory tests at diagnosis in 272 patients with AAD showed that 84% of patients presented with hyponatremia, 52% with elevated thyroid-stimulating hormone (TSH), but only 34% with hyperkalemia. In a clinical study, residual production of glucocorticoids was found in 58 of 192 patients with AAD, more common in men and associated with shorter disease duration. No differences in HRQoL scores, frequency of adrenal crisis, or glucocorticoid replacement doses were found. Baseline levels of cortisol and adrenocorticotropic hormone (ACTH) correlated with peak stimulated cortisol following injection with high-dose synthetic ACTH. A case-control study identified different levels of 19 biomarkers of CVD and inflammation (Olink) in 43 patients with AAD compared with 43 matched controls. Levels of receptor for advanced glycation end-product (RAGE) correlated with the frequency of adrenal crisis and HRQoL scores. Programmed cell death ligand 2 and leptin levels significantly declined following injection of high-dose synthetic ACTH in patients without residual glucocorticoid production.

In conclusion, findings in routine laboratory tests may point to undiagnosed AAD, especially unexplained hyponatremia and elevated TSH. Anticipating hyperkalemia might delay the diagnosis. Residual glucocorticoid production is common in AAD, especially in men, but the clinical relevance remains uncertain. Future work could assess any clinical or pathophysiological implications of altered biomarker profiles in AAD, including elevated RAGE, and direct effects of elevated ACTH.

Samandrag

Forsinka diagnose av autoimmun Addisons sjukdom (AAS) aukar risikoen for ei potensielt dødeleg binyrekrise. Ved etablert sjukdom er det stor variasjon i kliniske utfall, med forskjellar i risiko for binyrekriser, kardiovaskulær sjukdom og helserelatert livskvalitet. For høg og ufysiologisk tilførsel av glukokortikoid er tenkt å vere viktig årsaker, men kven av pasientane som er mest utsett for dårlegare kliniske utfall og kvifor er ikkje fullt ut forstått. Formålet med avhandlinga har vore å utforske moglege hint til tidleg diagnose og variasjonane i kliniske utfall ved AAS.

Retrospektiv gjennomgang av rutineblodprøver ved diagnosetidspunktet for 272 pasientar med AAS viste at 84 % hadde hyponatremi, 52% auka nivå av tyreoidea-stimulerande hormon (TSH), men berre 34 % hadde hyperkalemi. I ein klinisk studie hadde 58 av 192 pasientar med AAS bevart eigenproduksjon av glukokortikoid, meir vanleg blant menn og ved kortare sjukdomsvarigheit. Det var ingen forskjell i livskvalitet-skår, førekomst av binyrekrise eller glukokortikoid-doser mellom pasientar med og utan restproduksjon. Startverdiar av kortisol og adrenokortikotropt hormon (ACTH) korrelerte med oppnådd kortisol-verdi etter injeksjon av høg-dose syntetisk ACTH. Ein kasus-kontroll-studie fann ulike verdiar for 19 biomarkørar for kardiovaskulær sjukdom og inflammasjon (Olink) mellom 43 pasientar med AAS og 43 kontrollpersonar. Verdiar for reseptor for avanserte glykerte endeproduktar (RAGE) korrelerte med helserelatert livskvalitet og førekomst av binyrekrise. Verdiar av programmert celle-død ligand 2 og leptin fall signifikant etter injeksjon av høg-dose syntetisk ACTH blant pasientar utan restproduksjon av glukokortikoid.

Hovudkonklusjonane er at funn i rutineprøver kan gi hint om udiagnostistert AAS, særleg hyponatremi utan anna klar årsak og auka TSH. Forventa funn av hyperkalemi kan truleg bidra til å forsinke diagnosen. Restproduksjon av glukokortikoid er vanleg blant pasientar med AAS, særleg blant menn, men den kliniske verdien er uviss. Vidare arbeid kan utforske eventuell klinisk eller patofysiologisk betyding av avvikande biomarkørprofil for kardiovaskulær sjukdom og inflammasjon ved AAS, inkludert auka RAGE, og direkte effektar av auka ACTH.

List of Publications

Paper I: Sævik ÅB, Åkerman AK, Grønning K, Nermoen I, Valland SF, Finnes TE, Isaksson M, Dahlqvist P, Bergthorsdottir R, Ekwall O, Skov J, Nedrebø BG, Hulting AL, Wahlberg J, Svartberg J, Höybye C, Bleskestad IH, Jørgensen AP, Kämpe O, Øksnes M, Bensing S, Husebye ES. Clues for early detection of autoimmune Addison's disease - myths and realities. J Intern Med (2018) 283(2):190–9. doi: 10.1111/joim.12699

Paper II: Sævik ÅB, Åkerman AK, Methlie P, Quinkler M, Jørgensen AP, Höybye C, Debowska AJ, Nedrebo BG, Dahle AL, Carlsen S, Tomkowicz A, Sollid ST, Nermoen I, Grønning K, Dahlqvist P, Grimnes G, Skov J, Finnes TE, Valland SF, Wahlberg J, Holte SE, Simunkova K, Kämpe O, Husebye ES, Bensing S, Øksnes M. Residual corticosteroid production in autoimmune Addison disease. Journal of Clinical Endocrinology and Metabolism 2020. 105 2430–2441. (10.1210/clinem/dgaa256)

Paper III: Sævik ÅB, Grethe Ueland, Åkerman AK, Methlie P, Quinkler M, Jørgensen AP, Höybye C, Debowska AJ, Nedrebo BG, Dahle AL, Carlsen S, Tomkowicz A, Sollid ST, Nermoen I, Grønning K, Dahlqvist P, Grimnes G, Skov J, Finnes TE, Valland SF, Wahlberg J, Holte SE, Kämpe O, Husebye ES, Bensing S, Øksnes M. Altered biomarkers for cardiovascular disease and inflammation in autoimmune Addison's disease – a cross-sectional study. *Accepted in EJE in September 2023.*

Associated Work

Åkerman AK, Sævik ÅB, Thorsby PM, Methlie P, Quinkler M, Jørgensen AP, Höybye C, Debowska AJ, Nedrebo BG, Dahle AL, Carlsen S, Tomkowicz A, Sollid ST, Nermoen I, Grønning K, Dahlqvist P, Grimnes G, Skov J, Finnes TE, Valland SF, Wahlberg J, Holte SE, Kämpe O, Husebye ES, Øksnes, M Bensing S. Plasma metanephrines in patients with autoimmune Addison's disease with and without residual adrenocortical function. *Accepted in J Clin Med in May 2023.*

Ueland G, Methlie P, Heie A, Stokland AEM, Dahle AL, Sævik ÅB, Løvås K, Husebye ES. Profound Changes in Inflammatory and Cardiovascular Biomarkers in Patients with Autonomous Cortisol Secretion. *Accepted in EJE in June 2023*.

Didriksen NM, Sævik ÅB, Sortland LS, Øksnes M, Husebye ES. Sex-specific limitations in physical health in primary adrenal insufficiency. Front Endocrinol (Lausanne). 2021;12:718660.

Sævik ÅB, Wolff AB, Björnsdottir S, Simunkova K, Hynne MS, Dolan DWP, Bratland E, Knappskog PM, Methlie P, Carlsen Set al. Potential transcriptional biomarkers to guide glucocorticoid replacement in autoimmune Addison's disease. Journal of the Endocrine Society 2021 (10.1210/jendso/bvaa202)

Bothou C, Anand G, Li D, Kienitz T, Seejore K, Simeoli C, Ebbehoj A, Ward EG, Paragliola RM, Ferrigno R, Badenhoop K, Bensing B, Øksnes M, Esposito D, Bergthorsdottir R, Drake W, Wahlberg J, Reisch N, Hahner S, Pearce S, Trainer P, Etzrodt-Walter G, Thalmann SB, Sævik ÅB, Husebye ES, Isidori AM, Falhammar H, Meyer G, Corsello SM, Pivonello R, Murray R, Bancos I, Quinkler M, Beuschlein F. Current Management and Outcome of Pregnancies in Women with Adrenal Insufficiency: Experience From a Multicenter Survey. J Clin Endocrinol Metab (2020) 105(8):e2853–63. doi:10.1210/clinem/dgaa266

Endopodden - an educational podcast in endocrinology endopodden.podbean.com

Contents

1. Introduction

1.1 Anatomy and Physiology

1.1.1 Adrenocortical Steroids

Situated on top of the kidneys, the adrenal glands consist of an outer cortex and an inner medulla and are surrounded by a capsule of connective tissue (1). The main function of the adrenal cortex is to produce steroid hormones: mineralocorticoids (MCs), glucocorticoids (GCs), and adrenal androgens. The production of adrenocortical steroids is compartmentalized into three histologically distinct zones: with *de novo* synthesis of MCs in the outer zona glomerulosa, GCs in the middle zona fasciculata, and adrenal androgens in the inner zona reticularis (Figure 1). MCs and GCs are collectively called corticosteroids, and the main types in humans are aldosterone and cortisol (2). The main adrenal androgens are dehydroepiandrosterone (DHEA) and its sulfated form, DHEAS (3).

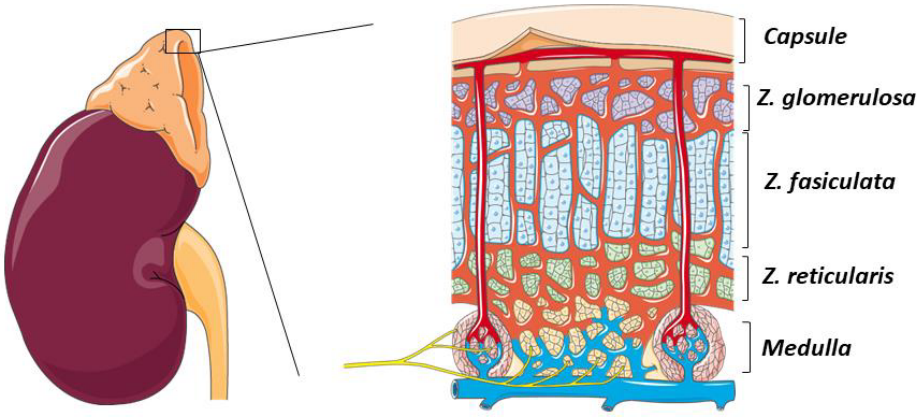


Figure 1. The adrenal gland consists of an outer cortex, an inner medulla, and a surrounding capsule. The adrenal cortex further consists of three histologically and functionally distinct zones (z.): z. glomerulosa, z. fasiculata, and z. reticularis. (The figure includes pictures from Servier Medical Art, licensed under a Creative Commons Attribution 3.0 unported License.)

A summary of adrenocortical steroidogenesis is depicted in Figure 2. Being steroid hormones, aldosterone, cortisol, and DHEAS share cholesterol as a precursor. The first modification of cholesterol to pregnenolone is also shared, but subsequent modifications differ between the zones, mainly facilitated by cytochrome P450 and hydroxysteroid dehydrogenase enzymes (4).

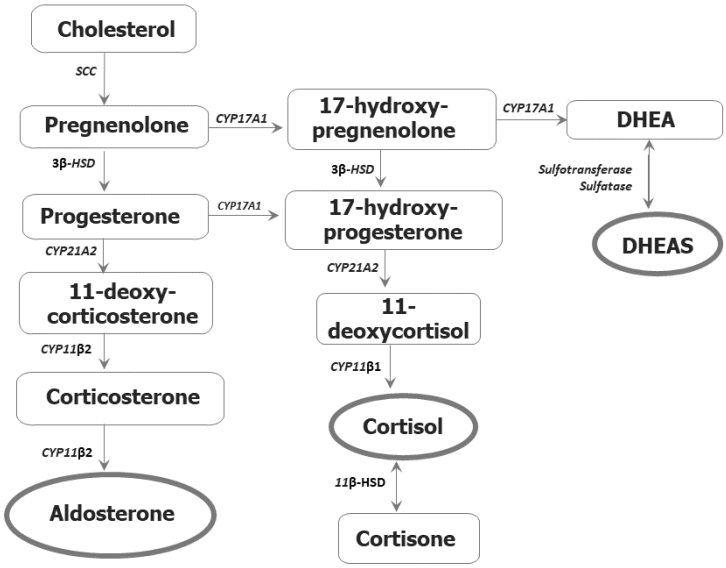


Figure 2. Adrenocortical steroidogenesis. The main adrenocortical steroids (aldosterone, cortisol, and dehydroepiandrosterone sulfate) are represented with bold circles and precursors and metabolites with rectangles. The arrows mark the direction of the enzymatic modification, catalyzed by side-change cleavage (SCC), cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzyme families, and (de)sulfation enzymes.

Adrenocortical steroids exert a multitude of effects in human health and disease. Aldosterone mainly works to maintain electrolyte and fluid homeostasis by increasing sodium and water reabsorption and potassium excretion in kidney nephrons (5). Cortisol regulates metabolic homeostasis, stress response, immunity, cognition, and cardiovascular function, amongst other effects (6). Due to its lipophilic nature, approximately 90% of circulating cortisol is bound to cortisol-binding globulin (CBG) (7). DHEA and DHEAS primarily serve as prohormones that can be converted to androgen receptor-binding steroids in target tissues (3).

1.1.2 ACTH

The production of cortisol is stimulated by adrenocorticotropic hormone (ACTH) and regulated by the hypothalamus-pituitary-adrenal axis. In unstressed conditions, ACTH cause cortisol levels to rise in the early morning and peak around the time of awakening, then decline throughout the afternoon and reach nadir at late night (8). This circadian rhythm is superimposed by ultradian oscillations, and cortisol levels can be further fine-tuned in response to physiological (e.g. infection) or emotional stress (6).

Specifically, circadian cues as well as perceived stressors spur the production of corticotropin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus, which incites the production of pro-opiomelanocortin (POMC) in the anterior pituitary (9). Next, prohormone convertases enzymatically process POMC to ACTH, which promotes the production of cortisol in the adrenal cortex. As a mechanism of negative feedback, sufficient or excess levels of cortisol lead to repressed expression of the *CRH* and the *POMC* genes. Opposite, low cortisol levels function as a positive feedback signal to ultimately increase cortisol biosynthesis (9).

ACTH belongs to a group of peptide hormones named melanocortins, which are characterized by the shared origin from POMC and overlapping binding to the five melanocortin receptors (MC1R to MC5R) (10). MC2R is specific for ACTH and mainly expressed in the adrenal cortex, but is present in other tissues as well, including adipocytes, urogenital tissue, immune cells, and vascular endothelial cells (10, 11). The ability of ACTH to activate the full range of melanocortin receptors implies that ACTH actions in health and disease may stretch beyond regulation of steroidogenesis, to possibly include regulation of skin pigmentation (MC1R), autonomic functions (MC3R), appetite (MC4R), exocrine secretions (MC5R), and immune response (MC1R-MC5R) (10, 12, 13). However, knowledge of any physiological relevance of extra-adrenal effects of ACTH *in vivo* is still limited due to difficulties in distinguishing between GC-dependent and GC-independent effects (14).

1.2 Autoimmune Addison's Disease

1.2.1 History and Epidemiology

In his landmark paper of 1855, the English physician Thomas Addison was the first to propose a disease due to adrenal gland failure, characterized by "*anemia, general languor, and debility, remarkable feebleness of the heart's action, irritability of the stomach, and a peculiar change of color in the skin*" (Figure 3) (15). Based on post-mortem examinations of 11 individuals, he suggested several etiologies later confirmed to cause Addison's disease, i.e. primary adrenal insufficiency (PAI), such as tuberculosis, cancer, and hemorrhage. He was particularly intrigued by a case of bilateral adrenal fibrosis that "*did not result as usual from a deposit either of a strumous or malignant character, but appears rather to have been occasioned by an actual inflammation – that inflammation having destroyed the integrity of the organs, and finally led to their contraction and atrophy*", probably being the first description of autoimmune Addison's disease (AAD) (15).

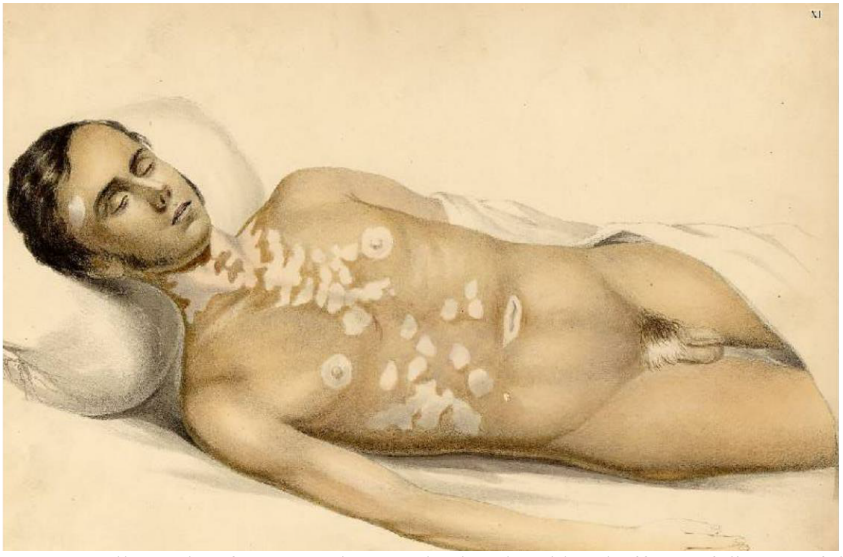


Figure 3. Illustration from "On the constitutional and local effects of disease of the supra-renal capsules" (1855) by Dr. Thomas Addison, depicting a deceased patient with hyperpigmented skin, vitiligo, and scant pubic hair. (Public domain provided by The University of Iowa Digital Libraries (16)).

Evidence to support the concept of autoimmune etiology came more than a century later when circulating autoantibodies against adrenal tissue were demonstrated in patients with PAI (17). In 1992, the steroidogenic enzyme 21-hydroxylase (21OH) was identified as the main target for the circulating adrenal autoantibodies (18).

In Europe, the 20th century brought forth a shift in the dominating cause of PAI (19). Following the rise of autoimmunity and the fall of tuberculosis, autoimmunity is now the dominating etiology of PAI and accounts for more than 80 % of cases among adults (20, 21). While still a rare disease, the prevalence of PAI seems to increase in European countries, with 39 cases per million inhabitants reported in the United Kingdom in the 1960s (22) compared to 144-221 cases per million in Nordic countries about 50 years later (20, 23). AAD is slightly more common in women, and although it can occur at any age, patients are on average 30-40 years old at the time of diagnosis (20, 21). AAD can occur as an isolated disease or as part of an autoimmune polyendocrine syndrome (APS) (24). APS1 is a rare, monogenic disease caused by mutations in the autoimmune regulator gene (*AIRE*) (25). More common, APS2 is considered the result of polygenic risk combined with environmental factors, clinically manifesting as AAD together with autoimmune thyroid disease and or type 1 diabetes mellitus (26).

1.2.2 Pathophysiology

Why and how AAD develops is not completely understood, but a hypothetical sequence of events is presented in the following. In genetically susceptible individuals, an environmental factor may cause adrenocortical tissue damage and leakage of 21OH (27). What this environmental factor might be is currently unknown, but suggestions include viral infections, stress, and pollutants (19). Another key question is what drives the infiltration of autoreactive immune cells into the damaged adrenocortical tissue. Possibly, adrenocortical cells may contribute to their own destruction by secreting chemokines as part of the local inflammatory response to tissue damage (27). Recruited immune cells may include autoreactive T cells specific towards 21OH, which drive the further destruction of adrenocortical tissue (27).

The pathophysiological contributions of autoreactive B cells are uncertain (27). While autoantibodies against 21OH (21OHab) are excellent markers of autoimmune etiology, they do not seem to partake directly in the destruction of adrenocortical tissue *in vivo* (27). For instance, 21OHab readily pass the blood-placenta-barrier, but there is no evidence of impaired adrenocortical function in babies carried by mothers with AAD (28). Still, titers of 21OHab are reported to correlate with the degree of adrenal insufficiency in preclinical disease and the risk of progression and remain elevated in established AAD (29). This raises another fundamental question of what fuels the autoimmune process to persist in longstanding disease, as one would expect the initial target to be depleted following years of autoimmune attack (29).

Post-mortem histopathological examinations of patients with AAD have found bilaterally atrophied adrenal glands, if grossly visible at all, but with (partly) spared adrenal medulla (30-33). Microscopic examination of the adrenal cortex may show widespread lymphocytic infiltration, interspersed with hyperplastic nodules, loss of the three-zonal architecture, and fibrosis (32-34).

1.2.3 Natural History

The natural history of AAD may differ between patients but is classically described as four stages with progressive loss of adrenocortical tissue and function, before manifesting as clinically overt AAD (Figure 4) (35-37). Approximately 0.5% of the healthy population are at stage 0, the only hint of any AAD risk being the presence of 21OHab (38). Estimations on how many proceed to stage 1 vary from 0-90%, where increased plasma renin activity or concentration and possibly reduced aldosterone levels provide the first biochemical signs of impaired adrenocortical function (36). At stage 2, morning cortisol and ACTH levels are still normal, but the cortisol response to a stimulation test with synthetic ACTH ($ACTH_{1-24}$) is blunted. Stage 3 is characterized by the addition of elevated ACTH and possibly hyperpigmented skin (36, 39). At stage 4, adrenocortical insufficiency is both clinically and biochemically evident, with low or undetectable levels of cortisol and aldosterone, and elevated levels of renin and ACTH (36). It has been suggested that at least 90 % of adrenocortical tissue must be destroyed before symptoms and signs emerge (40).

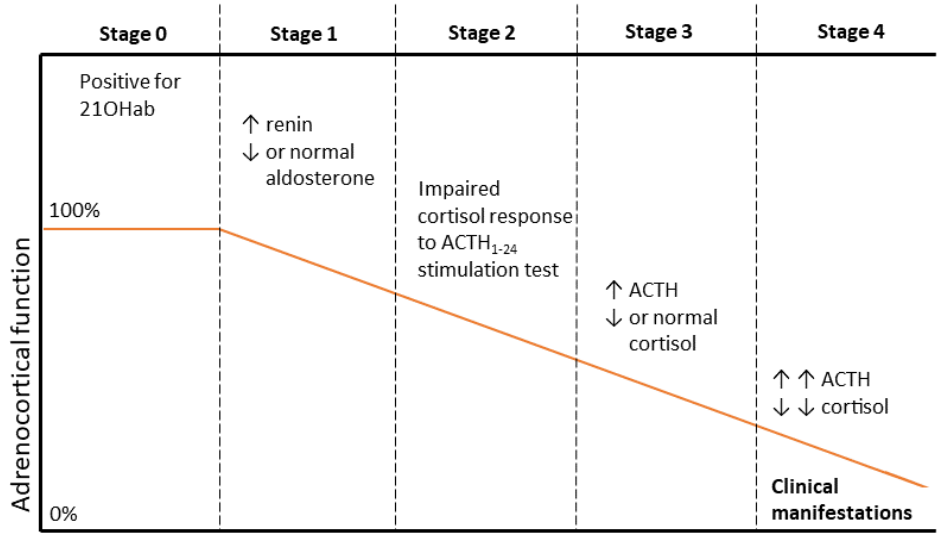


Figure 4. The natural history of AAD from subclinical to overt disease. (Modified with permission from the publisher (35)).

Emerging data have raised doubt that AAD development is a linear and irreversible process that inevitably results in the complete loss of all adrenocortical tissue and function. For one, not all adrenocortical zones are necessarily affected, evident by case reports of isolated MC deficiency in AAD, or preserved production of MCs but low levels of cortisol and adrenal androgens (37, 41).

In 1993, DeBellis et al reported biochemical normalization and loss of 21OHab-positivity in three individuals who initially had blunted response to the $ACTH_{1-24}$ stimulation test (stage 2) (42). Interestingly, all three individuals had received high-dose GC therapy for concomitant Graves ophthalmopathy, suggesting that the restoration of adrenocortical function could be caused by the high-dose GC therapy (42). Indeed, the potent immunomodulatory effects of GCs are postulated to underlie the frequent observation that zona fasciculata function is preserved longer than zona glomerulosa function in developing AAD (19).

Later case reports have suggested that spontaneous improvement of adrenocortical function might occur in longstanding AAD (37, 43-45). For instance, Smans and Zelissen described the case of a 46-year-old man with a 7-year history of AAD who

was able to discontinue all GC and MC replacement therapy and remained in excellent health five years later (45, 46). Intrigued by their finding, the authors set out to investigate possible adrenocortical recovery in 27 other patients with AAD (46). Although no new cases of adrenocortical recovery were identified, seven patients had detectable levels of morning cortisol despite having abstained from their usual corticosteroid replacement for more than a day, considered to reflect residual adrenocortical function (RAF) (46).

Recent data have indicated that RAF may be common in longstanding PAI. In 2019, Vulto et al found detectable levels of the GC precursor 11-deoxycortisol in 8 of 20 patients with a 15–20-year history of PAI (47). Soon after, Napier et al reported serum cortisol > 20 nmol/L in six of 37 patients with established AAD following 36 hours of GC withdrawal but found only a minor increase in cortisol levels following $ACTH_{1\text{-}24}$ stimulation testing (48).

1.2.4 Diagnosis

The clinical manifestations of AAD are mainly unspecific and have a gradual onset. The rarity of the disease may further contribute to a delayed diagnosis, leaving patients at risk of developing a potentially fatal adrenal crisis, but also impairing health-related quality of life (HRQoL) (49, 50). Studies on adrenal insufficiency in general have revealed that most patients are initially misdiagnosed, for instance with psychiatric or gastrointestinal illness (20, 49). Worryingly, a study on 216 patients in Germany found that 20% of patients had experienced symptoms and signs of adrenocortical insufficiency for 5 years before eventually being diagnosed (49), and a study on 60 patients with PAI in Poland found that > 40% were diagnosed following an adrenal crisis (50).

Common symptoms and signs of AAD include general malaise and fatigue, decreased appetite and unintended weight loss, nausea, abdominal and musculoskeletal pain, depression and anxiety, dizziness, and low blood pressure (24, 51). Still, a conspicuous craving for salt and an increased pigmentation of the skin and mucus membranes are distinctive features that may hint at the diagnosis (24).

Once suspected, the diagnosis of AAD is relatively easy to establish. The first step is to confirm the impaired adrenocortical function biochemically, which can be assessed in a morning blood sample before any hydrocortisone treatment is initiated. The combined finding of serum cortisol < 100 nmol/L and plasma ACTH at least two-fold elevated above the upper reference limit strongly indicates PAI (24). Important pitfalls include conditions that influence CBG concentrations and therefore measured cortisol levels, including an increase by estrogens and a decrease by inflammation (7).

Insufficient production of MCs manifests as low serum aldosterone and elevated plasma renin activity or concentration. Levels of adrenal androgens may be low as well (51). Other reported biochemical aberrations include low sodium, elevated potassium, hypercalcemia, changes in blood count (anemia, eosinophilia, lymphocytosis), hypoglycemia, and elevated liver transaminases (50-53).

Baseline biochemical testing is usually followed up by a confirmatory $ACTH_{1-24}$ stimulation test, also referred to as cosyntropin or synacthen test, and is considered the gold-standard method for diagnosing PAI (54). The stimulation test employs tetracosactide acetate (Synacthen®), a synthetic preparation of the first 1-24 of 39 amino acids of the endogenous ACTH peptide (51). Specifically, serum cortisol is measured before and 30 and 60 minutes after intravenous administration of 250 µg tetracosactide acetate. Using immunoassays, a normal response has been defined as peak cortisol exceeding 500 or 550 nmol/L, but updated cut-off levels for liquid-chromatography tandem-mass-spectrometry (LC-MS/MS) assays are lower; 412 or 486 nmol/L after 30 or 60 minutes, respectively (54).

After the diagnosis of PAI has been established, a final diagnostic step is to determine its etiology (24). An autoimmune cause can be evaluated by measuring 21OHab. If detected, the presence of concomitant autoimmune diseases should be considered, both at diagnosis and at annual follow-up visits. Suspicion of APS1 can be evaluated by measurement of interferon 1 autoantibodies and or sequencing of *AIRE* (25). If an autoimmune cause is not detected, a broader etiological search guided by clinical suspicion should be done (24).

1.2.5 Treatment

Current treatment strategies consist of daily replacement of adrenocortical hormones lacking – that is GCs, MCs, and occasionally DHEA – for life with the overall goal to restore patient health, well-being, and everyday normal functioning as well as avoid adrenal crisis (51).

Hydrocortisone or cortisone acetate tablets are the cornerstones of GC replacement in AAD, with only a minority using long-acting GCs (e.g. prednisolone, dexamethasone) (21, 55). The recommended daily dose is 15-25 mg of hydrocortisone equivalents, but higher doses are common (51, 55). The half-life of hydrocortisone and cortisone acetate is approximately 90 minutes, and the total dose is typically divided into two or three doses (19).

The dangers of too high GC exposure (e.g. worse cardiometabolic health) and too low (e.g. fatigue, adrenal crisis) are well-described, but there is currently no reliable tool to guide GC replacement doses. Recently suggested tools are cortisol concentrations in hair and gene expression levels, but these are not yet validated in AAD (56, 57). Instead, GC dose adjustments are primarily based on clinical judgment. Crude signs of overreplacement include weight gain, sleep disturbances, and peripheral edema. Underreplacement may manifest as unintended weight loss, fatigue, and general malaise (51).

Circadian misalignment of cortisol is considered to contribute to poor clinical outcomes in AAD as well (58). In recognition of this, new therapeutic options have been developed to better replicate the circadian rhythm of cortisol in patients with AAD, including modified-release hydrocortisone formulation (Plenadren®) and continuous subcutaneous hydrocortisone infusion (59, 60). In addition, a twice-daily modified-release hydrocortisone formulation (Efmody®) closer mimicking the early morning rise in cortisol has recently been made commercially available in Europe for the treatment of congenital adrenal hyperplasia (60, 61).

MC replacement typically consists of 0.05-0.1 mg fludrocortisone (e.g. Florinef®) daily, and patients are advised to not restrict their intake of salt (i.e. sodium chloride)

(21, 51, 62). Fludrocortisone has a mean half-life of nearly 5 hours, making once-daily administration sufficient (5).

International guidelines advise against routine DHEA supplementation due to the lack of clear benefits in clinical trials (3, 51). A 6-month trial of 25-50 mg DHEA may still be attempted in female patients experiencing depressive symptoms, low libido, and or persisting fatigue despite optimized GC and MC replacement therapy (51).

Adequate patient care in AAD does not simply consist of optimized pharmacological treatment but extends to repeated patient education. Important topics to address include background information on adrenal physiology and AAD pathophysiology, when and how much to increase GC replacement during intercurrent illness and stress, how to recognize and manage an incipient adrenal crisis, and any concerns the patient may have for everyday life and overall prognosis (63). Patients should be equipped with a steroid emergency card stating the necessity of prompt hydrocortisone administration upon severe illness or stress and offered practical training on how to self-administer an emergency injection of GCs (51). In Norway, the diagnosis of PAI and risk of adrenal crisis should be registered as «critical information» in the national summary care record to ensure prompt and appropriate action by healthcare professionals in any event of severe illness (64).

1.2.6 Clinical Outcomes

Available evidence and clinical experience suggest that AAD is a heterogeneous disease with large variations in clinical outcomes. For instance, there seem to be inter-patient differences in frequencies of adrenal crisis, life expectancy, cardiovascular risk, and HRQoL (65-67).

Adrenal Crisis

Adrenal crisis is the most feared complication of adrenal insufficiency and requires prompt recognition and treatment to avoid a lethal outcome (68). No established definition exists, but it is generally understood as an acute and severe health deterioration in a patient with adrenal insufficiency, with symptoms and signs of

corticosteroid deficiency that resolve following treatment with hydrocortisone and saline infusions (60, 68-71).

The pathophysiology of an adrenal crisis is incompletely understood, but in general relates to the relative or absolute lack of corticosteroids (Figure 5). It can be challenging to distinguish between an acute crisis and milder episodes of corticosteroid deficiency, as commonly shared features include fatigue and general malaise, gastrointestinal symptoms, electrolyte disturbances, hypotension, and postural dizziness. In severe cases, patients may develop hypovolemic shock. Clinical manifestations of infection are commonly present as well, as an infection is the number one precipitating cause of an adrenal crisis (72). Other precipitating causes may be emotional stress, surgery, strenuous physical activity, and missed GC doses (72).

Treatment of an adrenal crisis consists of high-dose hydrocortisone and saline fluid infusions, as well as targeted treatment of any precipitating illness, e.g. antibiotics for bacterial infections (73). If a diagnosis of adrenal insufficiency is not previously known, it is recommended that blood samples for analysis of cortisol and ACTH are obtained before initiation of hydrocortisone infusion, but only if it can be done without delaying the treatment (51).

In Europe, reported incidence rates of adrenal crises in PAI range from 5 to 17 per 100 patient-years, with part of the variation likely relating to different definitions (74-76). The frequency seems to be unevenly distributed, with a subset of patients suffering repeated crises while possibly half of patients never experience any (74). The reasons for the great variation in patient susceptibility to an adrenal crisis are inadequately understood, but a higher risk has been linked to a history of previous crisis, higher age, and the presence of autoimmune comorbidity (73). Recently, Quinkler et al found that patients with PAI who experienced adrenal crisis had significantly higher serum potassium and lower serum sodium levels compared with patients without adrenal crisis, but no significant differences in weight-adjusted GC or MC replacement doses were found (77).

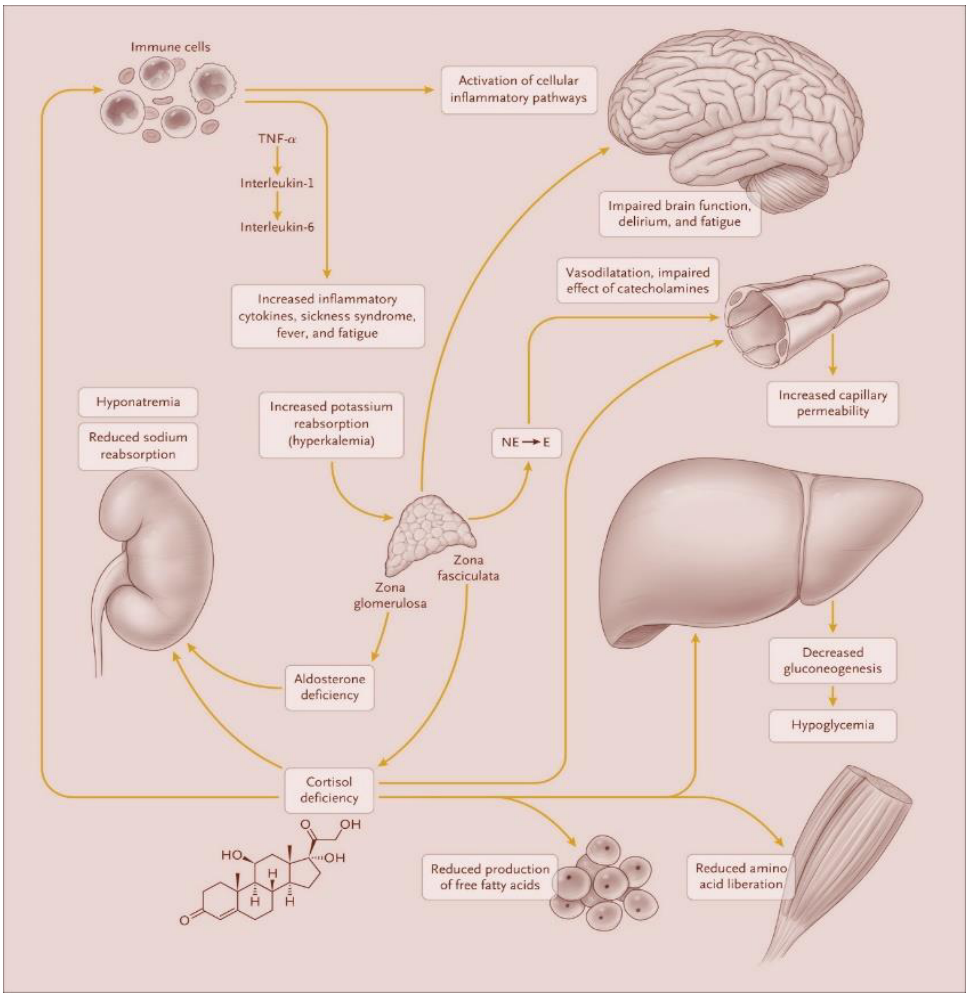


Figure 5. Pathophysiological model of adrenal crisis. (Reprinted with permission from the publisher (68)).

Mortality

It is beyond doubt that corticosteroid replacement saves lives in PAI, but studies suggest that mortality rates remain increased even after initiation of treatment. For instance, data from Sweden have demonstrated more than two-fold higher death rates in patients with PAI in general and with AAD specifically compared with population controls, with cardiovascular disease (CVD) being the leading cause of death (78, 79). Similarly, a recent study found nearly 2-fold higher mortality rates for ischemic heart disease among patients with PAI compared with population controls in the

United Kingdom (80). In contrast, a Norwegian study found excess death rates caused by adrenal crisis, infections, and sudden death in patients with AAD diagnosed before the age of 40 only, and otherwise normal mortality rates (66). Possible explanations for the inconsistent findings may include methodological differences as well the trend for lower GC replacement doses over the past decades (66, 81). Otherwise, it is not clear whether the increased mortality rates for CVD mainly reflect a higher prevalence of CVD or rather increased case-fatality rates (82). Indicative of the latter, Skov et al found ischemic heart disease events to have a greater 30-day case fatality rate in patients with AAD compared with population controls in Sweden (82).

Cardiovascular Risk

Concern has been raised for cardiometabolic health in AAD as several studies have shown more CVD and CVD risk factors in patients with adrenal insufficiency (80, 82-87). Both too high GC doses and unphysiological GC replacement are commonly put to blame (54, 88, 89), and clinical trials have demonstrated improvement in several metabolic parameters following a switch from conventional hydrocortisone or cortisone acetate to modified-release hydrocortisone in patients with adrenal insufficiency (85, 90). However, not all studies find excess CVD risk or any correlations to GC replacement doses (54), as further outlined below. The discrepancies in results suggest that a subgroup of patients might be at particular risk of CVD and that factors beyond GC replacement might play a role as well.

Focusing on AAD, a Swedish registry study found increased prescription rates for antihypertensive drugs, diuretics, and lipid-lowering agents in AAD compared to population controls, but the finding was only significant for younger age groups (86). A second Swedish registry study even found a significantly lower frequency of hypertension and obesity in AAD compared with population controls, and no difference for hyperlipidemia or type 2 diabetes, although blood pressure levels positively correlated with GC replacement doses (21). A third study found more ischemic heart disease in AAD compared with Swedish population controls and increasing risk with higher GC and MC replacement doses, but the findings were only significant in women (82).

Few clinical studies have addressed cardiometabolic health in patients with AAD compared with controls. While an Italian study on 39 patients with AAD found impaired glucose tolerance, hypercholesterolemia, and hypertriglyceridemia in patients compared with controls (87), another study on 63 patients with AAD in Poland found no differences in levels of adiponectin or leptin between patients and controls (91).

Looking to PAI, a clinical study on 147 patients in Sweden and South Africa reported higher levels of triglycerides, low-density lipoprotein, and C-reactive protein (CRP) and lower levels of high-density lipoprotein in patients compared with controls (84). Only levels of high-density lipoprotein significantly correlated with hydrocortisone replacement doses. Of note, hypertension, diabetes mellitus, and the use of lipid-lowering therapy were more common among patients than controls, but patients had significantly lower body mass index (BMI) (84). Likewise, Bergthorsdottir et al later reported higher levels of triglycerides, lower levels of high-density lipoprotein, more diabetes mellitus, antihypertensive medication, and lipid-lowering therapy in 76 patients with PAI compared with controls in Sweden (83). The patients also had a higher frequency of metabolic syndrome, but a similar extent of visceral adipose tissue, and neither could be linked to the hydrocortisone replacement doses. No difference was found for levels of CRP or adiponectin, but the study revealed altered biomarker profiles of CVD in patients compared with controls, including elevated levels of interleukin 6 (IL6) (83).

Epidemiological and clinical studies have demonstrated strong and consistent associations between CVD risk and inflammatory markers, including higher levels of CRP and IL6 (92, 93). In line with this, chronic inflammation is considered to contribute to increase CVD risk in patients with autoimmune diseases (94, 95). In a recent mapping of CVD incidence in 19 common autoimmune diseases, each disease was associated with increased CVD risk, on average corresponding to the risk caused by type 2 diabetes and increasing with the number of autoimmune comorbidities (94). The second highest frequency was noted for AAD, with nearly three times more CVD than population controls. Importantly, the excess risk could not be explained by traditional risk factors such as age, sex, socioeconomic status, blood pressure, BMI,

smoking, dyslipidemia, or type 2 diabetes, pointing to autoimmunity as an independent risk factor for CVD. Important to note, the study did not account for any impact of inflammatory modulators, including GC therapy (94). In theory, both the autoimmune process and unphysiological GC replacement could contribute to higher CVD risk by altering inflammation in AAD (83, 96), but insight into the inflammatory state in AAD is currently restricted to reports of elevated levels of selected cytokines (97-100).

Any impact of deviant levels of MCs, adrenal androgens, or ACTH on CVD risk in AAD have rarely been explored. Well-known from hyperaldosteronism, excess MC is associated with impaired cardiometabolic health (5, 101), but a Swedish registry study found no associations between daily fludrocortisone dose and prevalence of hypertension, hyperlipidemia, or type 2 diabetes in patients with AAD (21). Lack of adrenal androgens could potentially impair cardiovascular health in AAD as well, but DHEA replacement is not found to have a clear impact on lipid profiles or endothelial function (82). Finally, ACTH could in theory affect cardiometabolic health by modulating inflammation, energy homeostasis, and blood pressure in a GC-independent manner (10, 14). ACTH is reported to induce human adipocyte expression of IL6 and monocyte chemoattractant protein 1 (MCP1), which are both linked to inflammation and cardiometabolic risk (102). In contrast, a clinical study found ACTH to increase blood pressure in healthy normotensive and hypertensive individuals, but not in two patients with Addison's disease, suggesting that the hypertensive effect of ACTH is GC-dependent (103).

HRQoL

HRQoL seems to be impaired in patients with PAI, but large interindividual differences exist, evident by the wide ranges in scores to both generic (e.g. Research and development 36-Item, RAND-36) and disease-specific (e.g. AddiQoL-30) HRQoL questionnaires (20, 104, 105). Patient characteristics associated with better or worse HRQoL are only partly identified.

A recent mapping of HRQoL in 529 patients with adrenal insufficiency in the United States identified several modifiable factors to be associated with poorer physical and

or mental HRQoL, including higher GC replacement doses, co-morbidities related to GC excess, higher financial burden, difficulties in self-management, and lack of family support (106). Several, but not all, clinical trials have further demonstrated improved HRQoL following a switch from conventional GC replacement to modified-release hydrocortisone or continuous subcutaneous hydrocortisone infusion in patients with adrenal insufficiency (90, 107, 108). Otherwise, a German study on 110 patients with Addison's disease found that a higher number of subjective precrises were significantly associated with lower AddiQoL-30 scores (109).

A recent study by our research group identified sex-specific limitations in HRQoL in PAI, with perceived physical health being the most affected in female patients but social functioning in male patients compared with normative data (104). Regarding any impact of autoimmune etiology, the same study found higher HRQoL in patients with AAD compared with non-autoimmune PAI but also higher HRQoL in patients without concomitant autoimmune disease (104). The findings could be partly confounded by the greater preponderance of women with autoimmune comorbidity, as women in general report lower HRQoL than men.

2. Rationale and Aims of the Thesis

Health threats that remain for patients with AAD include delayed diagnosis and adverse clinical outcomes.

Specifically, delayed diagnosis of AAD prolongs the burden of corticosteroid deficiency and increases the risk of premature death (49, 50). The thought that routine laboratory tests may provide hints to spur suspicion of AAD is therefore appealing. While such findings are frequently listed in the literature and textbooks, they have not been subject to systematic audit.

In established AAD, excess and unphysiological corticosteroid replacement likely contributes to adverse clinical outcomes but does not seem to fully explain the reported variation in risk of adrenal crisis, excess CVD, and impaired HRQoL (67, 77, 104). Patient characteristics that might be associated with better or worse outcomes and why are not fully known.

Emerging evidence suggests that some corticosteroid production might persist in established AAD, but how common it is or any associations with clinical outcomes are not known. Likewise, inconsistent findings emphasize the need for a better understanding of what drives CVD risk in AAD and which subgroups of patients may be most vulnerable. Whether markers of CVD and inflammation could provide clues to clinical outcomes as well or be modulated by elevated ACTH has not been studied.

The overall aim of this thesis has been to explore clues to early diagnosis and the variation in clinical outcomes in patients with AAD. In response to the outlined gaps in knowledge, the thesis is based on three papers with the specific aims to

- Assess findings in routine laboratory tests at diagnosis of AAD (**Paper I**)
- Assess the frequency and clinical features of residual corticosteroid production in AAD (**Paper II**)
- Assess biomarker profiles of CVD and inflammation in patients with AAD compared with controls, any associations with clinical outcomes, and GC-independent effects of elevated ACTH (**Paper III**)

3. Material and Methods

3.1 Study Participants, Design, and Endpoints

Paper I was a retrospective registry audit of findings in routine laboratory tests at diagnosis of AAD in 272 patients with AAD in Norway (n=137) and Sweden (n=135). All patients were enrolled in the Norwegian Registry for Organ-specific Autoimmune Diseases (ROAS) or the Swedish Addison Registry. The diagnoses were established between 1978 and 2016 for the Norwegian cohort and between 2000 and 2016 for the Swedish cohort. Autoimmune etiology was ensured by only including patients with presence of 21OHab and chronic use of GC and MC replacement following diagnosis. The primary endpoint was laboratory values noted at the time of diagnosis before the initiation of corticosteroid replacement. The secondary endpoint was any difference in laboratory values between patients with adrenal crisis compared with patients without an adrenal crisis at diagnosis.

Paper II was a two-staged clinical study of patients with verified AAD who received follow-up at one of the participating 17 study centers in Norway, Sweden, and Germany. During 2018-2019, a total of 197 patients were initially included of which five did not wish to proceed to blood sampling while 192 completed study participation. The diagnosis of AAD was confirmed by the presence of 21OHab as well as chronic use of GC and MC replacement. The primary endpoint was the number of patients with endogenous corticosteroid production. Secondary endpoints included associations between endogenous corticosteroid production and age, sex, disease duration, adrenocortical steroid replacement doses, frequency of adrenal crisis, BMI, blood pressure, and HRQoL, as well as any change in cortisol levels following injection of 250 µg $ACTH_{1-24}$.

Paper III was a cross-sectional case-control study, including 43 patients from **Paper II** and 43 healthy controls from a previous clinical study by our research group (54) that were matched for age (by decade), sex, and BMI (±1 kg/m^2). Among patients with AAD, 23 had residual production of GCs and 20 did not, in the following

referred to as with or without RAF. The primary endpoint was differences in biomarker profiles of CVD and inflammation between patients and matched controls. Secondary endpoints included sex-specific differences in biomarker profiles, correlations between biomarker levels and frequency of adrenal crisis and AddiQoL-30 scores, differences in biomarker profiles between patients with and without RAF, and changes in biomarker profiles following injection of 250 µg $ACTH_{1-24}$. Being GC-depleted, any change in biomarker profiles in patients without RAF served as a model for GC-independent effects of increasing ACTH from high (baseline) to very high levels (after $ACTH_{1-24}$ stimulation).

A summary of the study designs is given in Figure 6.

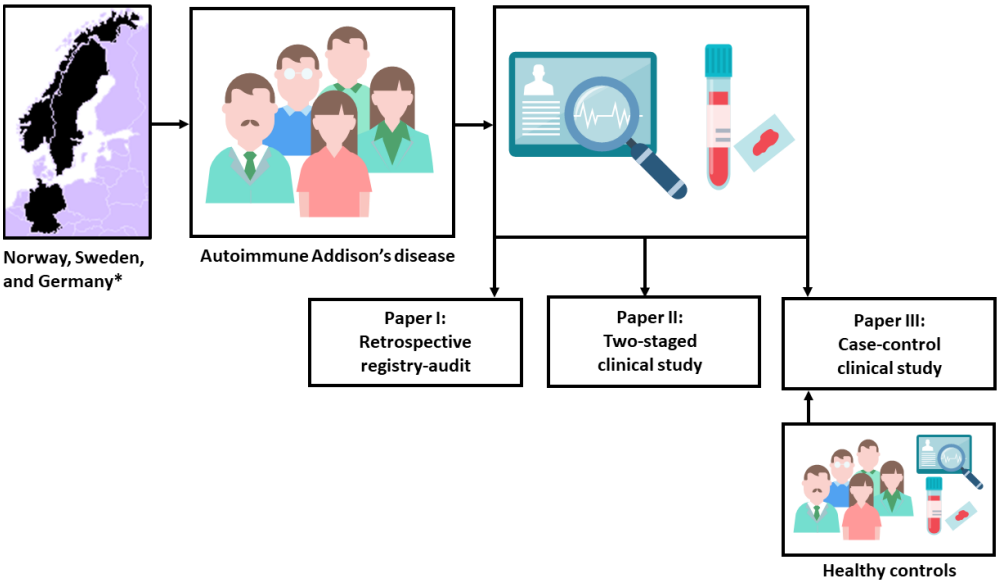


Figure 6. (The figure includes pictures from ConceptDraw and Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License.) *Patients from Germany were included in **Papers II** and **III**.

3.2 Definitions

3.2.1 Biomarkers and Clinical Outcomes

In **Papers II** and **III**, biomarkers and clinical outcomes were assessed as measures of health, disease, or response to interventions. Biomarkers are defined as quantifiable indicators of biological processes, such as genetic, biochemical, or physiologic features. Instead, clinical outcomes reflect how an individual feels, functions, or survives, and may be assessed by the clinician, the patient, another observer (e.g. caregiver), or be a test score. Tools for assessment may for instance be questionnaires, reported number of events, or standardized tasks (110, 111).

3.2.2 Adrenal Crisis

In **Paper I,** an adrenal crisis was defined by the following criteria: acute hospital admission, systolic blood pressure < 100 mmHg, and by clinical judgment considered to be an adrenal crisis. The cut-off for systolic blood pressure was set to 100 mmHg as it is a frequent feature in suggested definitions (69, 72, 112).

In **Papers II** and **III**, the criteria for an adrenal crisis were an acute hospital admission and treatment with intravenous infusion of hydrocortisone. The number of adrenal crises in the past year was reported by the patients and not cross-checked against medical records.

3.2.3 Residual Corticosteroid Production

In **Papers II** and **III**, residual corticosteroid production was defined as the presence of the main GC (cortisol > 0.914 nmol/L) and or MC (aldosterone > 8 pmol/L) together with their respective precursors, 11-deoxycortisol (> 0.144 nmol/) and corticosterone (> 0.144 nmol/L). The cut-off levels equaled the lower limits of quantification on a LC-MS/MS method developed in-house and previously described in detail (113).

3.3 Clinical Data

Data on age, sex, and BMI were noted for all study participants in **Papers I-III,** and for all patients with AAD, presence of 21OHab.

In **Paper I**, we systematically noted patient data from the time of diagnosis and before the initiation of corticosteroid replacement, including if the patient had been admitted acutely to the hospital, any autoimmune comorbidity, use of levothyroxine, blood pressure measurements, and in the Swedish patient cohort, hyperpigmentation. Patients were subgrouped as with or without adrenal crisis at diagnosis.

In **Paper II,** we used a structured history form to collect clinical data (included in the Appendix). Noted data included disease duration, any comorbidities, types and doses of adrenocortical steroid replacement, use of other medications, number of infections and adrenal crises in the past year, and blood pressure measurements. Patients further completed one generic (RAND-36) and one disease-specific (AddiQoL-30) questionnaire assessing HRQoL (included in the Appendix).

Data on the number of adrenal crises in the past year and AddiQoL-30 scores were included in **Paper III** as well.

3.4 Laboratory Assessment

3.4.1 Paper I

For each patient, we systematically noted patient characteristics recorded at the time of diagnosis before the initiation of corticosteroid replacement. Patient characteristics included sex, age, if the patient had been admitted acutely to the hospital, 21OHab, any autoimmune comorbidity, use of levothyroxine, height, weight, blood pressure, and in the Swedish patient cohort, hyperpigmentation. An adrenal crisis was defined by the following criteria: acute hospital admission, systolic blood pressure < 100 mmHg, and by clinical judgment considered to be an adrenal crisis.

Likewise, laboratory data were systematically noted for each patient at the time of diagnosis and before the initiation of corticosteroid replacement. The only exception was proven 21OHab, which in most cases was analyzed after the diagnosis of PAI had been established. The laboratory analyses included serum sodium, serum potassium, blood hemoglobin, serum alanine aminotransferase, serum calcium, serum creatinine, serum glucose, serum thyroid-stimulating hormone (TSH), thyroid peroxidase (TPO) autoantibodies, random serum cortisol, stimulated serum cortisol, plasma ACTH, serum aldosterone, plasma renin activity or concentration, and serum DHEAS.

Although the laboratory methods varied between study centers and during the 38-year study period, we chose to keep the given values. When reference ranges diverged profoundly between study centers, as was the case for TSH, we dichotomized values to elevated or not elevated based on the different upper reference limits.

3.4.2 Paper II

Following informed consent, patients returned on an agreed morning at 8 am for blood sampling (Stage 1) after abstaining from cortisone acetate or hydrocortisone and fludrocortisone for at least 18 and 24 hours, respectively. As an extra safety measure upon GC withdrawal, patients were non-fasting. Routine blood tests were analyzed by the local laboratories, and in addition, a serum sample was sent to Haukeland University Hospital, Bergen, Norway, and stored at -80°C before analysis of adrenocortical steroids, including precursors and metabolites, by a LC-MS/MS method developed in-house and previously described in detail (113).

All patients with residual production of corticosteroids and 20 patients without were invited to Stage 2. On an agreed morning, patients returned for a 250 µg $ACTH_{1-24}$ stimulation test, again upon withdrawal of GC and MC replacement. Blood samples were obtained before and 60 minutes after the injection of $ACTH_{1-24}$.

3.4.3 Paper III

All patients (except one) and controls had gone through a morning $ACTH_{1-24}$ stimulation test, with serum samples collected before and 60 minutes after the

injection of $ACTH_{1-24}$. Serum samples from patients and matched controls were analyzed for 177 unique biomarkers included in the Cardiovascular II (CVD II) and Inflammation panels by Olink (Uppsala, Sweden). The samples had been stored at -80°C and were shipped on dry ice. The Olink method is based on proximity extension assay technology, described in detail elsewhere (114, 115). The biomarker unit is given as normalized protein expression (NPX), which is an arbitrary unit on a Log2 scale (114, 115). Biomarker values below the lower limit of quantification were included, as recommended by Olink (116). Initially, 44 patients and 44 healthy controls were included, but one sample (patient without RAF) did not pass the preanalytical quality control by Olink, and this patient was removed from the dataset together with its matched control before the statistical analyses.

3.5 Statistics

The sample size calculation for **Paper II** was based on a previous report of residual GC production in 2 of 13 patients with AAD (117) and was found to require 139 study participants. For **Paper III**, sample sizes were calculated with the Olink Power Tool (https://olinkproteomics.shinyapps.io/Power_Tool/), estimating that 44 patients and 44 controls were required for the comparison of 177 biomarkers.

In **Papers I-III**, demographical data were given as median, mean, or percentage along with a variability measure. Any between-group differences were evaluated by an independent T-test, Mann-Whitney U test, or Chi-square test, as appropriate, and any temporal changes by paired samples T-test or Wilcoxon signed rank test, depending on the distribution of data. Pearson's or Spearman's correlation coefficient (r) was calculated for correlation analyses. Results from binary logistic regression analyses in **Paper II** were presented as an odds ratio with 95 % confidence intervals.

In general, the significance threshold was set to the conventional $P<0.050$. Acknowledging the risk of false positives upon multiple testing, the more conservative $P<0.010$ was used for the analysis of most secondary endpoints in

Paper I and all analyses in **Paper II**. In **Paper III,** a false discovery rate (FDR) of 5% with the Benjamini-Hochberg method was employed, if not otherwise stated.

3.6 Ethics

The studies were conducted in agreement with the Declaration of Helsinki and ICH-GCP guidelines. Ethical approval was obtained, data protection officers consulted, and written informed consent was ensured before participant enrolment. (**Paper I:** ROAS permit no. 2013/1504/ REK vest, Swedish Addison Registry permit no. 2008/296-31/2. **Papers II** and **III**: Norway permit no. 2018/751/REK sør-øst and REK 2016-00174, Sweden permit no. 2018/2247-32, Germany permit no. Eth-47/18). **Papers II** and **III** were also registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT03793114).

4. Summary of Results

4.1 Paper I

The studied cohort consisted of 272 patients with AAD, of which 173 were women and 99 were men. Age at diagnosis ranged from 5 to 79 years, with a median of 36 years. Most patients were diagnosed during acute hospital admission (69%), and one-third fulfilled the pre-defined criteria of an adrenal crisis. Just over half of the patients (54 %) had at least one other autoimmune disease.

Other clinical findings at diagnosis included systolic blood pressure <100 mmHg in 42 %, diastolic blood pressure <60 mmHg in 26 %, and underweight defined as BMI below 18.5 kg/m^2 in 24% of patients. In the Swedish patient cohort, 87% had hyperpigmented skin.

Among routine laboratory tests, hyponatremia defined as serum sodium < 137 mmol/L was the most common deviation, present in 207 of 247 (84%) patients. Hyperkalemia, defined as serum potassium >5.0 mmol/L, was found in 82 of 242 (34%) patients and was accompanied by hyponatremia in all but one patient. Twenty-one patients (9%) had serum sodium and potassium values within the respective reference ranges.

On group level, electrolyte disturbances were more distinct in patients with adrenal crisis, with median serum sodium of 127 mmol/L [101–138] and median serum potassium of 5.0 mmol/L [3.5–8.4] compared with 132 mmol/L [103–142] (P<0.001) and 4.5 mmol/L [3.2–8.6] (P<0.001), respectively, in patients without adrenal crisis.

Among the 153 patients who did not use levothyroxine, 79 (52%) had elevated TSH. Measurement of TPO autoantibodies was only recorded for 22 patients and found present in eight of them. There was a small but significant negative correlation between TSH and random cortisol (r=-0.248, N=138, P=0.003).

A few individuals presented with aberrant glucose, hemoglobin, alanine aminotransferase, calcium, and creatinine levels, but the mean values were all within their respective reference ranges.

Serum cortisol was generally low (median 62 nmol/L [1–668]) but significantly lower in patients with adrenal crisis (38 nmol/L [2–442]) compared with patients not in crisis at diagnosis (81 nmol/L [1–668], P<0.001). $ACTH_{1-24}$ stimulation test failed to increase cortisol above 500 nmol/L in all but one patient, a 27-year-old woman with confirmed use of oral contraceptive pills. The median plasma ACTH level was 278 pmol/L ([1-1910]), corresponding to the upper reference range limit in most assays. Measurement of DHEAS was given for 33 patients and below 2 μmol/L in all but two of them.

4.2 Paper II

58 of 192 (30.2%) patients had residual production of GCs, of which 24 patients had residual production of MCs as well, and two other patients had residual MC production only. Despite a preponderance of women in the patient cohort (60.4%), more than half of the patients with residual GC production (33 of 58) were men. Residual GC production was more common in shorter disease duration (median 6 [0-44] vs. 13 [0-53] years, P<0.001), but was even found in patients diagnosed several decades ago. Similarly, residual MC production was associated with shorter disease duration (median 5.5 [0.5-26.0] vs. 13 [0-53] years, P<0.004), but also with lower fludrocortisone replacement dose (median 0.075 [0.050-0.120] vs. 0.100 [0.028-0.300] mg, P=0.005) and higher plasma renin concentration (median 179 [22-915] vs. 47.5 [0.6-658.0] mU/L; P<0.001)

In contrast, there were no significant differences in the frequency of adrenal crisis, infections, presence of concomitant autoimmune diseases, presence of symptoms of GC or MC deficiency, hydrocortisone equivalent doses, or clinical features (e.g. BMI, blood pressure, HRQoL scores) between patients with or without residual production of GCs and or MCs. Patients with residual GC production had lower levels of ACTH

compared with patients without GC production, but this was not statistically significant (median 123 [26-278] vs. 147 [1-278] pmol/L, P=0.087).

Fifty-five patients with residual GC production underwent an $ACTH_{1-24}$ stimulation test. Quantifiable serum cortisol and 11-deoxycortisol were replicated in all but five patients, and these five patients were excluded from the statistical analyses. In the remaining 50 patients with residual GC production, the median peak cortisol was 75 nmol/L [9-419] after the $ACTH_{1-24}$ stimulation, confirming the diagnosis of adrenal insufficiency.

Baseline levels of cortisol and ACTH strongly correlated with peak stimulated cortisol levels ($r = 0.989$, $P<0.001$, and $r=-0.487$, $P<0.001$, respectively).

4.3 Paper III

The patient and control groups each consisted of 43 participants, of which 19 were women, and the participants had a mean age of 40 years and a mean BMI of 24 kg/m². Except for shorter disease duration, there were no significant differences in baseline characteristics (i.e., age, sex, BMI, blood pressure, concomitant autoimmune diseases, hydrocortisone equivalent doses, fludrocortisone doses) between patients with and without RAF ($P>0.05$).

Nineteen of the 177 biomarkers significantly differed between patients and controls ($P<0.050$, FDR 5%), namely: IL6, MCP1, receptor for advanced glycation end products (RAGE), adrenomedullin, galectin 9, tumor necrosis factor receptor superfamily member 9, receptor activator of nuclear factor kappa-B and its ligand, death receptor 4 and 5, lymphotactin, P-selectin glycoprotein ligand 1, spondin 2, fibroblast growth factor 23, interleukin 12B, matrix metallopeptidase 12, sulfotransferase 1A1, fibroblast growth factor 21 (FGF21), and T-cell surface glycoprotein (CD4). The greatest difference in NPX was found for FGF21 (difference 0.8 NPX), and all biomarkers but one biomarker (sulfotransferase 1A1) were elevated in patients.

Higher levels of RAGE were associated with more adrenal crises (r = 0.415, P = 0.006) and lower AddiQoL-30 scores (r=-0.347, P=0.028). The frequency of adrenal crisis also correlated with levels of CD4 (r=0.338, P=0.029) and FGF21 (r=-0.317, P=0.041) (correlations not corrected for multiple testing).

For subgroup analyses, female patients had significantly higher levels of 8 biomarkers compared with female controls (IL6, MCP1, galectin 9, spondin 2, death receptor 4, placenta growth factor, RAGE, and tumor necrosis factor receptor superfamily member 9). In contrast, there were no significant differences in biomarker levels between male patients and male controls, or between patients with and without RAF (P<0.050 and FDR 5%).

In patients without RAF, levels of programmed cell death ligand 2 (PDL2) and leptin significantly declined 60 minutes after injection of $ACTH_{1-24}$ compared with baseline levels (-0.15 NPX, P=0.0001, and -0.25 NPX, P=0.0003, respectively).

5. Discussion

5.1 Discussion of the Main Results

5.1.1 Laboratory Findings at Diagnosis

In **Paper I**, we found hyponatremia to be present in most patients at diagnosis. The fact that low sodium is the most frequent electrolyte disturbance in clinical practice (118) and that most patients without adrenal crisis only had mild hyponatremia add to the challenge of early diagnosis in AAD. Still, the prevalence of hyponatremia increases with age, and it is less common in the 30 to 50 year-age-group when AAD typically emerges (118). We therefore suggest that an unexplained low sodium value in younger patients who present with unspecific symptoms and signs could warrant consideration of undiagnosed AAD.

Suspicion of AAD could be further spurred by the additional finding of elevated TSH, as found for half of the patients who did not have known thyroid disease. Of note, there is a well-known link between primary hypothyroidism and hyponatremia as well (119, 120). However, both the frequency and the degree of hyponatremia seem to be lower in patients with primary hypothyroidism compared with the findings at diagnosis of AAD given in **Paper I**.

Several mechanisms may contribute to increased levels of TSH in patients with untreated AAD. For one, it could reflect an undiagnosed, concomitant autoimmune thyroiditis, but we were hindered from evaluating this in most patients as any measurement of TPO autoantibodies had not been recorded. Alternatively, elevated TSH could have resulted from the lack of inhibition by cortisol, as known from the literature and here implied by the negative correlation between levels of cortisol and TSH. Thyroid hormone, on the other hand, induces cortisol metabolism (74). The clinician should therefore beware that a patient with unspecific health complaints and elevated TSH who deteriorates after initiation of levothyroxine treatment for assumed hypothyroidism might instead have undiagnosed AAD (74, 121).

Contrary to widespread opinion, hyperkalemia was only found in one-third of patients, and other non-hormonal routine laboratory tests were largely normal as well. Nearly one in ten patients had normal values of both serum sodium and serum potassium at diagnosis, highlighting that electrolyte disturbances are not mandatory in untreated AAD. A high potassium value, especially if accompanied by low sodium, would still strengthen the suspicion of AAD. The degree of hyponatremia and hyperkalemia further seem to reflect the severity of the disease, found to be significantly more distinct in patients with adrenal crisis.

Beyond findings in routine laboratory tests, 87% of the Swedish patients were considered to have hyperpigmented skin at diagnosis. This is somewhat higher than the reported frequency of 74% with hyperpigmentation at diagnosis in a previous Norwegian study (20), but both figures illustrate how an unexpected darkening of the skin may be a valuable clue to the diagnosis.

We found low cortisol, low aldosterone, low DHEAS, high ACTH, and high plasma renin activity or concentration to be consistent findings in untreated AAD. Of note, we found that normal serum cortisol at baseline or after the ACTH stimulation test could not be used to exclude AAD, illustrated by three patients presenting with baseline cortisol levels exceeding 500 nmol/L and in one of them, peak stimulated cortisol of 703 nmol/L. Two of the patients used oral contraceptive pills, and an actual hypocortisolism was therefore likely camouflaged by increased CBG, further indicated by a mere 35 nmol/L increase in cortisol following $ACTH_{1\text{-}24}$ stimulation in one of them. The third patient was probably diagnosed at an early stage, due to concomitant diabetes mellitus type 1 and primary hypothyroidism, and likely had residual GC production.

5.1.2 Residual Corticosteroid Production

In **Paper II**, we found residual production of GCs in 30 % and residual production of MCs in 14 % of patients with AAD. The observation that residual production of GCs was more common than residual production of MCs corresponds to the classical natural history of AAD, with impaired zona glomerulosa function typically preceding zona fasciculata impairment (19). It is currently not known where in the body the

residual corticosteroid production occurs, but it could hypothetically arise from remnant adrenocortical stem cells that are spared from the autoimmune attack, ectopic adrenocortical tissue, or reflect a compensatory increase in local corticosteroid synthesis in various organs (2, 122, 123).

Against our hypothesis, we were not able to demonstrate any link between residual corticosteroid production and clinical outcomes, including frequency of adrenal crisis or HRQoL. While such links may not exist, the lack of significant associations could also relate to methodological limitations, as further discussed below.

We were surprised by the clear preponderance of men among patients with residual GC production. Possible mechanisms for this are currently not known but could relate to sex-specific differences in general immunology and or adrenal biology (124, 125). Specifically, animal models indicate that turnover rates for adrenocortical tissue differ between the sexes, three times faster in females compared to males, postulated due to the inhibitory effects of high androgen levels (124-126). Epidemiological studies further demonstrate a female predominance in most adrenocortical diseases, including AAD, adrenal Cushing's syndrome, mild autonomous cortisol secretion, *KCNJ5* mutations in aldosterone-producing adenomas, and adrenocortical carcinoma (127-129). In addition, there are fundamental differences in immune function between women and men, driven by genetic, hormonal, and environmental factors (130). In general, women mount stronger immune responses than men, yielding better protection against pathogens but also higher susceptibility to autoimmune diseases (130). Taken together, one could speculate that sex-specific differences in adrenocortical biology and or a weaker autoimmune attack could contribute to more adrenocortical tissue being spared in men with AAD compared with women, possibly underlying the higher frequency of residual GC production in men. This hypothesis should be further investigated in the future.

We found residual production of both GCs and MCs to be significantly associated with shorter disease duration, but importantly, present in patients diagnosed several decades ago as well. The wide range in disease duration indicates great heterogeneity in the natural history of AAD after diagnosis, as suggested by others as well (43-46,

48). Looking to other autoimmune diseases may provide clues to how and why residual production of corticosteroids may be possible even in longstanding AAD. For instance, it may be that the autoimmune attack in AAD wax and wane as in Graves' disease and multiple sclerosis, allowing for intermitting regeneration of functional adrenocortical tissue (122). In type 1 diabetes, however, the autoimmune destruction of pancreatic β cells is considered to be constant, but interestingly, persistent secretion of C peptide has been described in approximately one-third of patients (131), and even here more common in men and shorter disease duration (132).

The finding that patients with residual production of MCs had significantly lower doses of fludrocortisone could indicate less need for MC replacement. While elevated plasma renin concentration was an expected finding given the withdrawal of fludrocortisone, the significantly higher levels in patients with residual production of MCs hypothetically suggest that an activated renin-angiotensin-aldosterone-system may stimulate MC production in patients with remnants of zona glomerulosa tissue.

Hypothetical implications of residual corticosteroid production extend to the prospect of stimulatory treatment to improve adrenocortical function. In line with previous studies, we found only a limited increase in cortisol levels following $ACTH_{1-24}$ stimulation in patients with residual GC production (46, 48). Still, there seemed to be a close connection between the degree of residual GC production and any stimulatory potential, indicated by the strong correlations between higher baseline cortisol levels and peak stimulated cortisol, as seen in **Paper I** as well. Indeed, in clinical trials to improve adrenocortical function in AAD with repeated tetracosactide injections and or B-cell-depleting therapy, transient or lasting improvement has mainly been found in patients with the highest levels of baseline or peak stimulated cortisol at study entry (117, 133, 134).

5.1.3 Biomarkers of CVD and Inflammation

In **Paper III**, we identified 18 biomarkers of CVD and inflammation that were significantly higher and one biomarker significantly lower in patients with AAD compared with matched controls. The 19 biomarkers included apoptotic agents

(galectin 9, death receptor 4 and 5 (135, 136)), inflammatory agents (IL6, MCP1, RAGE, lymphotactin, CD4, tumor necrosis factor receptor superfamily member 9, interleukin 12b (135, 137-142)), regulators of bone homeostasis (receptor activator of nuclear factor kappa-B and its ligand, fibroblast growth factor 23 (143)), a vasoactive agent (adrenomedullin (144)), and modulators of general physiological processes, such as metabolism (FGF21 (145)), tissue remodeling (matrix metallopeptidase 12 (146)), cell adhesion (P-selectin glycoprotein ligand 1, spondin 2 (147, 148)), and sulfation (sulfotransferase 1A1 (149)).

Several of these biomarkers have previously been linked to the pathogenesis of autoimmune, cardiovascular, and metabolic diseases. For one, the single most deviant biomarker in AAD compared with controls, FGF21, is reported to predict future risk and progression of cardiometabolic diseases as well as overall mortality (145). On the other hand, clinical trials have demonstrated improved cardiometabolic outcomes following injection of FGF21, suggesting that FGF21 may increase as a compensatory response to excess metabolic stress and or represent FGF21 resistance (145, 150). Beyond roles in cardiometabolic risk, FGF21 has been implicated in the development and renewal of adrenocortical tissue, and experimental data suggest that injection of recombinant FGF21 could stimulate cortisol secretion in mice (151).

There is a well-established association between elevated IL6 and CVD risk, with mounting evidence from large-scale human studies even pointing to causality (152, 153). In this regard, it is interesting to note that elevated IL6 seems to be a consistent finding in PAI regardless of any recent intake of corticosteroid replacement or food (83, 89) even though both cortisol exposure and food intake have been reported to significantly influence IL6 levels (89, 154).

It is increasingly being recognized that women and men differ concerning epidemiology, pathophysiology, clinical manifestations, treatment response, and prognosis in CVD (155). In AAD, the excess risk of CVD seems to be sex-specific as well, implied by a Swedish population-based study finding significantly higher incidence rates of ischemic heart disease in women but not men with AAD compared with their sex-specific controls (82). We were therefore intrigued to find a similar

pattern of sex-specific difference in biomarker profiles, with eight biomarkers significantly higher in female patients and controls, but no significant difference in any biomarker levels between male patients and controls. It has been suggested that women with AAD may be more prone to GC overreplacement, possibly contributing to the sex-specific differences in CVD susceptibility (82). More autoimmune comorbidity in women with AAD could theoretically play a role as well, in line with the recent finding that CVD risk cumulatively increases with the number of autoimmune diseases (94). In **Paper II**, however, there was no significant difference in hydrocortisone equivalent doses per kg or body surface area, and while there was a higher proportion of women with APS2 compared with men, it was not significantly different (data not shown). Taken together, future research should seek to identify which factors underly the sex-specific difference in CVD risk in AAD and further explore any pathophysiological roles of the eight biomarkers we found elevated in women with AAD.

We were further intrigued by the significant associations between levels of RAGE, CD4, and FGF21 and frequency of adrenal crisis and, for RAGE, AddiQoL-30 scores as well. Future longitudinal studies are needed to assess their potential as markers of disease severity or any pathophysiological contribution to the interindividual variation in clinical outcomes in AAD. Nevertheless, we find it plausible that levels of inflammatory markers could be linked to clinical outcomes, as seen for a broad range of other conditions (156) and suggested in adrenal insufficiency by others as well. Specifically, Ekman et al found negative correlations between levels of C-X-C motif chemokine 11 and HRQoL subscores for general and physical health in 15 patients with AAD (100), and Isidori et al demonstrated significant associations between levels of soluble CD16 and metalloproteinase domain-containing protein 17 and the number of infections in 89 patients with adrenal insufficiency (85).

Hypothesizing that inflammatory biomarkers could provide hints as to why a subgroup of patients with AAD preserve some adrenocortical function, we compared biomarker profiles between patients with and without RAF, but no significant differences were identified. We cannot exclude that the analysis may have been

influenced by an insufficient number of patients in each subgroup and or the inclusion of very low cortisol levels in the definition of RAF.

In support of extra-adrenal effects of ACTH, we found that levels of PDL2 and leptin significantly declined following an increase in ACTH levels from high to very high in patients without RAF.

We are not aware of other studies suggesting a regulatory role of ACTH on PDL2. Being ligands to the same PD1 receptor, PDL1 and PDL2 both regulate inflammation, but some functional differences exist, partly due to the higher binding affinity but more restricted expression of PDL2 compared with PDL1 (157). In the context of autoimmune diseases, PDL1 variants have been implied in AAD risk (158), but any links to PDL2 variations have not yet been established.

We are not the first to suggest an inhibitory role of ACTH on leptin secretion (159), but whether this may influence appetite regulation in AAD is uncertain. The lack of a significant correlation between ACTH and leptin levels at baseline in patients without RAF might suggest that ACTH exerts a short-term regulatory effect on leptin levels that is lost in chronically elevated levels. In line with this, a previous study on 63 patients with AAD found no significant correlations between levels of ACTH and leptin in morning serum samples during GC withdrawal (91).

5.2 Methodological Considerations

5.2.1 Study Design and Study Participants

A principal strength of the present work is the focus on autoimmune etiology, ensured by only including patients with 21OHab and chronic use of GC and MC replacement medication. Of note, autoimmunity is only one of several possible etiologies of adrenal insufficiency and is less common than an iatrogenic cause (i.e. following prolonged, high-dose GC therapy) or pituitary disease (24). Important shared traits across disease etiologies include the vital need for GC replacement, and in PAI, MC replacement as well, but fundamental differences in pathophysiology and comorbidities imply that what is true for one etiology may not be generalizable to

others. While studies including multiple etiologies of adrenal insufficiency may better reflect the real-life experiences of endocrinologists, the abovementioned differences may influence study results and complicate the interpretation. Still, studies on non-autoimmune AI are often cited in the context of AAD, pointing to the need for more AAD-specific research (59, 82).

Other general strengths include the well-characterized patient cohorts and the high number of participating study centers. Including patients from several different geographical sites (**Papers I-III**) and from different time periods (**Paper I**) contributed to improving the generalizability of the findings. In **Papers I** and **II**, selection bias was reduced by inviting all patients who fulfilled the inclusion criteria at the participating study centers. Employed in **Paper I**, ROAS and the Swedish Addison Registry are estimated to include approximately 65% and 50% of patients with AAD in Norway and Sweden, respectively (21, 160). **Papers I-III** were further designed to avoid any influence of adrenocortical steroid replacement on laboratory findings, by noting laboratory values before initiation of adrenocortical steroid replacement in **Paper I** and after > 18 hours of withdrawal in **Papers II** and **III**.

Important limitations apply as well. In **Paper I**, the retrospective design yielded limited control with reported data. For instance, not all laboratory values of interest had been recorded for every patient, and the time point of blood sampling was not always provided. Moreover, there was some variation in the laboratory methods and reference ranges used by the participating study centers and in the different decades. To partly adjust for this, we chose to dichotomize some of the laboratory values to elevated or not elevated.

In **Papers II** and **III**, study participants were prospectively included, but the cross-sectional study design hindered any evaluation of longitudinal trends. In **Paper II**, quantifiable levels of cortisol or aldosterone with its precursor were verified in the later $ACTH_{1-24}$ stimulation test, indicating that residual production of adrenocortical steroids might be a persistent phenomenon, but the significant association to shorter disease duration suggests that levels decline over time. While Napier et al demonstrated a rapid decline in adrenal steroidogenesis the first month after initiation

of replacement therapy in AAD (48), the natural history of residual adrenocortical function in longstanding AAD remains unknown. The cross-sectional study design was an even greater issue in **Paper III**, as we could not assess any predictive capacity of deviant biomarker levels on CVD risk. We were further hindered from evaluation of any short- or long-term effects by different doses of GC, MC, or DHEA replacement on biomarker profiles.

5.2.2 Definitions

Adrenal Crisis

Despite its severity, there is no universal definition of adrenal crisis, and no available clinical tool to detect its emergence or presence. While suggested definitions generally require an acute and severe deterioration in health status, additional definition criteria range from none at all to detailed clinical and biochemical features, expected time frame for improvement following parenteral GC administration, as well as grading systems relating to the extent of health care required for the adrenal crisis to resolve, e.g. outpatient care or hospital admission, or whether the adrenal crisis had deadly outcome (60, 68-71).

In **Paper I**, a retrospective audit of recorded data hindered firm adherence to a detailed definition of adrenal crisis. Instead, we chose a pragmatic definition based on frequently suggested features of adrenal crisis and the information available: acute hospital admission, systolic blood pressure < 100 mmHg, and on clinical judgment considered to be in an adrenal crisis. Thirty-three percent of the patients fulfilled all three pre-defined criteria. However, this may have been an underestimate as 70 % of the patients were diagnosed during an acute hospital admission, but half of them failed the criterion of systolic blood pressure under 100 mmHg and were therefore not defined as in adrenal crisis. For instance, two of the patients who were diagnosed during an acute hospital admission had low serum cortisol (<1 and 50 nmol/L, respectively) and severe electrolyte disturbances (serum sodium 103 mmol/L and serum potassium 8.6 mmol/L, respectively), but systolic blood pressure above 100 mmHg. One could speculate that the number of patients considered to suffer an adrenal crisis might have been higher if relative hypotension (i.e. systolic blood

pressure more than 20 mmHg lower than usual) had been included in the definition, as suggested by Rushworth et al (68), but this information was not available.

In **Papers II** and **III**, the number of adrenal crises in the past year was given by the patient. Even though the structured history form defined an adrenal crisis as an acute hospital admission with intravenous infusion of hydrocortisone, the given number was not cross-checked against medical records. This may have caused the number of adrenal crises to be erroneous for several reasons, including recollection bias and different perceptions of what an adrenal crisis is (109). Indeed, in a prospective study on 110 patients with AAD in Germany, Meyer et al found ten cases of adrenal crises that fulfilled the predefined criteria, but a three times higher frequency of patient-perceived crises, which were defined as subjective pre-crises (109). Specifically, the study found frequencies of 10.9 adrenal crises per 100 patient years but 33.4 cases of subjective pre-crises (109). In comparison, we found frequencies of 18.8 and 32.6 adrenal crises per 100 patient years **Papers II** and **III**, respectively, suggesting that the given numbers are overestimates or rather reflect subjective pre-crises. Compared with overt adrenal crises, subjective pre-crises likely pose less of a threat to health and life, but may still be clinically relevant, as indicated by the significant association to lower HRQoL found by Meyer et al (109). It is further possible that a subjective precrisis could develop into an overt adrenal crisis if the patient does not increase the GC replacement medication.

of a consensus definition for adrenal crisis constitutes a major methodological issue in research on adrenal crisis in general and on incidence rates in particular (70). While a strict and detailed definition may be ideal for research purposes, there might be a need for a broader, clinical definition as well to ensure timely recognition and treatment of an adrenal crisis even if not all stringent criteria are met. Further work is also needed to evaluate any clinical relevance of subjective pre-crises in AAD and how these should be managed.

Residual Corticosteroid Production

Previous studies on preserved production of corticosteroids in PAI have applied different terminology, methodology, and cut-off levels (46-48, 117, 134, 161).

In **Paper II**, we used the term "residual corticosteroid production", defined as quantifiable levels of both cortisol and 11-deoxycortisol for residual GC production and both aldosterone and corticosterone for residual MC production. The >18 and >24 hours withdrawal of GC and MC replacement, respectively, was considered sufficient for medication wash-out and still short enough to avoid any adrenal crisis, a fundamental ethical premise for the conduction of the study. Still, we found low but detectable levels of cortisol in several patients, likely representing incomplete elimination of hydrocortisone or cortisone acetate from the previous day. The usefulness of including the GC and MC precursors in the definitions was further illustrated by the measurement of serum cortisol of 797 nmol/L but undetectable 11-deoxycortisol levels in one of the study participants. The finding raised suspicion about the intake of GC replacement medication before blood sampling, which was later confirmed by the patient. Moreover, it served to increase our confidence that the conversion of 11-deoxycortisol to cortisol by 11β-hydroxylase is unidirectional, as reported in the literature (162, 163).

The strong correlations between levels of cortisol and 11-deoxycortisol and levels of aldosterone and corticosterone in **Paper II** might imply that measurement of the precursors could be sufficient for the evaluation of residual production of GCs or MCs in AAD. In line with this, Vulto et al found detectable 11-deoxycortisol (> 0.025 pmol/L) in 8 of 20 patients with PAI shortly after intake of their morning hydrocortisone dose (47). In the study by Vulto et al, any residual production of MCs was assessed as well, with corticosterone >0.17 pmol/L detected in 7 of 20 patients with PAI. Importantly, the blood samples had been obtained shortly after patients had taken their morning corticosteroid replacement doses, indicating that medication withdrawal may not be mandatory when focusing on corticosteroid precursors to assess endogenous corticosteroid production in PAI.

The low limits of quantification allowed by the LC-MS/MS assay contributed to the relatively high proportion of patients defined to have preserved production of GCs and or MCs. Changing the definition criteria to stimulated peak cortisol > 100 nmol/L decreased the prevalence to 10 % in **Paper II**, which is closer to the 14 % found by

Napier et al (48). While even a low degree of residual corticosteroid production is an interesting finding in itself, it is unlikely to be clinically relevant and might have contributed to the lack of significant associations with clinical outcomes, e.g. HRQoL or frequency of adrenal crisis. However, post-hoc analyses requiring higher levels of cortisol or aldosterone, e.g. within normal reference ranges, could neither detect significant differences in clinical outcomes.

Acknowledging the disadvantages of diverse terminology, we decided to adopt the abbreviation "RAF" in **Paper III**, as used by others. The term "residual adrenal function" might date back to 1953, in a paper on the clinical course following adrenal resection in patients with severe hypertension (164). It was later applied by Gan et al to describe peak stimulated cortisol above 400 nmol/L in two patients with AAD following regular injections of $ACTH_{1\text{-}24}$ (117). In 2020, the same researchers introduced the abbreviation "RAF" and extended the definition to include serum cortisol levels of 99 mmol/L or more while abstaining from any corticosteroid replacement (161). To emphasize the focus on the adrenal cortex, we chose to write it out as "residual adrenocortical function" instead of the more general "residual adrenal function".

To summarize, it is not currently evident what might be the best definition of RAF to be applied in future studies. While consensus on the terminology could be relatively easy to establish, more work is needed to determine whether the definition should include the precursor and or the main corticosteroid only, whether to use baseline or peak stimulated values, and importantly, what cut-off levels should be applied.

5.2.3 $ACTH_{1\text{-}24}$ Stimulation Test

In line with established clinical practice and international guidelines, we found the $ACTH_{1\text{-}24}$ stimulation test to be useful for diagnosing and verifying PAI in **Papers I** and **II**, respectively. However, the test may have been less suited for the evaluation of residual GC production (**Paper II**) or any GC-independent impact of ACTH on biomarker profiles of CVD and inflammation (**Paper III**).

As expected, baseline levels of ACTH were already elevated given the 24-hour withdrawal of any GC replacement in **Papers II** and **III**. It is therefore possible that the stimulatory potential and any GC-independent effects of ACTH had already been maximized before the $ACTH_{1-24}$ stimulation test. This could contribute to explain the limited increase in cortisol in patients with residual GC production in **Paper II**, as found by others as well (46, 48), and the significant decline of only two of 177 biomarkers in patients without residual GC production in **Paper III**.

Secondly, the injected dose of 250 µg tetracosactide represents a markedly supraphysiological ACTH exposure (165), making it even more difficult to evaluate any clinical relevance of the observed decline in PDL2 and leptin levels. In addition, the chronically elevated ACTH in AAD might have contributed to the lack of significant change in levels of IL6 and MCP1 in **Paper III**, as seen *in vitro* (102).

5.2.4 Biomarker Analyses

A great advantage of proteins as biomarkers relates to their executing roles in biological processes. Profiling of circulating proteins may therefore provide insight into the pathophysiology of both cardiovascular and autoimmune diseases and holds the potential to identify candidate targets for future therapeutic interventions in AAD (166, 167). Historically, large-scale profiling of proteins (i.e. proteomics) in serum has been hampered by technical limitations of the applied assays, including low sensitivity, low specificity, low multiplex capacity, and high sample volume requirements (166). The PEA technology by Olink is demonstrated to overcome these challenges (168) motivating the specific choice to analyze the CVD II and Inflammation biomarker panels in an exploratory study on possible mechanisms for cardiovascular risk in AAD and any links to clinical outcomes (**Paper III**).

Only one study has previously compared proteomic markers of CVD in patients with PAI and matched controls (83). Analyzing the CVD II and Inflammation panels further allowed for the attempt to validate some of the previous findings in **Paper III.** Specifically, the two panels included 34 biomarkers that had been analyzed in the previous Swedish study on 76 patients with PAI compared with controls, including

12 of the 17 markers found to significantly differ (83). However, we could only replicate elevated IL6. While both the Swedish study and **Paper III** demonstrated significant differences in levels of adrenomedullin and metalloprotease 12, the direction of difference was the opposite: found higher in patients by us but lower in patients in the other study. The discrepancies in results could partly relate to the difference in GC exposure between the two studies. Indeed, patients in **Paper III** had abstained from any corticosteroid replacement for at least 18 hours before the morning blood sampling, but in the Swedish study, patients had taken their morning hydrocortisone replacement shortly before the blood sampling (83).

Several methodological aspects beyond GC exposure may have influenced the findings in **Paper III**. For one, the high number of participating research centers may have contributed to more preanalytical error due to variation in sampling, handling, and storing of samples, even though we had developed written instructions to standardize study procedures across all centers (included in the Appendix). Otherwise, the final sample sizes of patients and controls were one short compared with the calculated requirement of 44 participants in each group, due to the removal of the patient sample (and corresponding control sample) that did not pass the preanalytical quality control.

Other limitations relate to missing data. Although patients had normal levels of cholesterol, triglycerides, and HbA1c, this information was not available for the healthy controls, and we did not have measurements of CRP for any study participant. Even though a previous study could not find any difference in levels of CRP between patients with PAI and controls, it is an accessible marker of inflammation and CVD risk (92, 93) and would therefore have been relevant for **Paper III** as well. More importantly, we did not have data on several conventional risk factors for CVD, including smoking history in patients (all controls were non-smokers). We further lacked data on the menopausal state, but the proportion of women being pre- and post-menopausal was probably similar among patients and controls as they were matched for age and only one patient had premature ovarian insufficiency.

An important issue in biomarker research in general relates to the high risk of false positive findings associated with multiple testing. Among suggested methods for correction, the FDR described by Benjamini-Hochberg is considered particularly appropriate for exploratory biomarker research and was therefore applied (169).

We cannot exclude that 60 minutes was too short to detect all effects of $ACTH_{1-24}$ injection on biomarker levels. Indeed, the half-lives of the analyzed biomarkers are largely unknown, and possible longer half-lives may have contributed to the lack of significant change in levels for some of the biomarkers. It is further possible that food intake might have confounded the results, as food intake has been reported to significantly influence several of the analyzed biomarkers, including a decline in leptin levels (62). This was less of a concern for the baseline comparison of biomarker profiles between patients with AAD and healthy controls, as all study participants had been instructed to eat before the morning blood sampling.

Last, but not least, any clinical implications of the biomarker findings in **Paper III** are uncertain. While the inclusion of matched controls allowed us to identify biomarkers that were altered in patients with AAD, the relative quantification and lack of reference ranges for the biomarkers hindered the evaluation of any clinical relevance of the detected statistical differences. In addition, the significant findings included both known and exploratory markers of CVD and inflammation. Scrutiny of the peer-reviewed literature provided insight into possible roles of the altered biomarkers in human health and disease, but for most of the biomarkers, high-quality evidence was scarce. The presented findings are therefore best considered candidate discoveries, useful for hypothesis generation, but require future validation and evaluation of any clinical implications.

6. Conclusion

This thesis has explored clues for early diagnosis and the variation in clinical outcomes in AAD. The main conclusions are:

In untreated AAD, hyponatremia is common, but hyperkalemia is only present in one-third of patients. Elevated TSH is present in half of the patients without known thyroid disease. Thus, unexplained hyponatremia, especially if accompanied by elevated TSH, should prompt consideration of AAD and lead to the paired analysis of morning cortisol and ACTH.

Residual GC production is present in one-third of patients with AAD, more common in men and shorter disease duration, but any clinical relevance remains to be proven. Higher levels of endogenous GCs are associated with greater response to $ACTH_{1\text{-}24}$ stimulation, and further work could explore any potential to exploit this therapeutically.

Biomarkers of CVD and inflammation differ between patients with AAD and matched healthy controls, especially in women. RAGE might be a marker of disease severity, associated with the frequency of adrenal crisis and HRQoL. Injection of $ACTH_{1\text{-}24}$ reduces levels of PDL2 and leptin in a GC-independent manner, but any clinical or pathophysiological relevance is uncertain.

7. Future Perspectives

Above all, continuous effort should be made to disseminate evidence-based knowledge on AAD, to increase awareness and diagnostic vigilance among healthcare professionals, and assist self-management among patients with AAD.

While the present work does not provide *the* answers to why clinical outcomes vary in AAD, it provides a basis for further work to explore the nature, clinical relevance, and therapeutic opportunities of RAF and altered biomarker profiles in AAD.

For RAF in AAD, compelling remaining questions include: Who are the patients with preserved GC production in longstanding disease? Where in the body is it located? Is there a true male preponderance, and why? What may be the clinical implications?

To assess any role of genetic factors, we could make use of the recent genome-wide association study in AAD performed by our research group and close collaborators (170). As most patients in **Paper II** were included in both studies, data could be merged to look for genetic variants that may be linked to RAF and identify potential causal mechanisms.

To understand the natural history of RAF in AAD, a prospective study could expand on the recent work by Napier et al showing a substantial drop in peak stimulated cortisol one month after the initiation of GC replacement (48). Specifically, a future study could map longitudinal changes in corticosteroid levels from diagnosis on and explore any associations with patient characteristics, levels of inflammatory markers, or clinical outcomes. The findings could further be adjusted for sex to assess the indicated differences between women and men with AAD concerning the frequency of RAF and altered biomarker profiles.

Nuclear imaging could be a useful tool in the search for the anatomical location(s) of residual corticosteroid production, but it would require a tracer with high adrenocortical uptake and low uptake in other tissues. The recently developed para-chloro-2-[18F]fluoroethyletomidate seems to meet these requirements (171), and Swedish research partners are currently planning to assess it in a follow-up study on selected patients with residual GC production in AAD.

While the large patient cohort in **Paper II** was sufficiently powered to assess the prevalence of residual GC production, an even larger cohort might be needed to determine whether higher levels of endogenous GCs are associated with clinical outcomes or could allow for a reduction in GC replacement doses. In addition, clinical trials should continue to explore whether a greater GC response to ACTH stimulation can be exploited therapeutically to improve adrenocortical function in AAD, as indicated by previous work (117, 133, 134).

Furthermore, there is a need for a better understanding of what drives CVD risk in AAD, and which subgroups of patients may be more susceptible. To address this, a national registry on Addison's disease could be coupled to national registries on CVDs and prescription databases to provide updated estimates on CVD incidence rates and drug-prescription patterns, verify the sex-specific difference in risk of ischemic heart disease found in Sweden (82), and identify other patient characteristics associated with more CVD in AAD. Next, a prospective case-control study could compare biochemical data between patients with AAD who have developed CVD and patients who have not. Candidate biomarkers for CVD, including biomarker findings in **Paper III**, could be analyzed in serum samples obtained one, five, or ten years ago, or at the time of diagnosis, to evaluate any predictive capacity for CVD risk.

An important future task is to establish consensus definitions of adrenal crisis and RAF in AAD. For adrenal crisis, two definitions may be required; one stringent definition to be used for research purposes and one broader definition to aid timely recognition and adequate management among patients, relatives, and health care professionals in general. For RAF, consensus definitions should be made for residual production of corticosteroids in general (i.e. RAF) and GCs and MCs separately. The definitions should state the recommended terminology, the biochemical criteria, and how it should be assessed. Specifically, it should establish which corticosteroids should be measured (e.g. precursors only or the main corticosteroid as well), the use of baseline or peak cortisol levels following $ACTH_{1\text{-}24}$ stimulation, and ideally, differentiate between detectable and clinically significant levels of corticosteroids.

8. References

1. Megha R, Wehrle CJ, Kashyap S, Leslie SW. Anatomy, Abdomen and Pelvis, Adrenal Glands (Suprarenal Glands). StatPearls. Treasure Island (FL): StatPearls Publishing

2. Taves MD, Gomez-Sanchez CE, Soma KK. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. American journal of physiology Endocrinology and metabolism. 2011;301(1):E11-24.
3. Wierman ME, Kiseljak-Vassiliades K. Should Dehydroepiandrosterone Be Administered to Women? The Journal of clinical endocrinology and metabolism. 2022;107(6):1679-85.
4. Pihlajoki M, Heikinheimo M, Wilson DB. Regulation of Adrenal Steroidogenesis. In: Levine AC, editor. Adrenal Disorders: Physiology, Pathophysiology and Treatment. Cham: Springer International Publishing; 2018. p. 15-66.
5. Esposito D, Pasquali D, Johannsson G. Primary Adrenal Insufficiency: Managing Mineralocorticoid Replacement Therapy. The Journal of clinical endocrinology and metabolism. 2018;103(2):376-87.
6. Lightman SL, Birnie MT, Conway-Campbell BL. Dynamics of ACTH and Cortisol Secretion and Implications for Disease. Endocr Rev. 2020;41(3).
7. Verbeeten KC, Ahmet AH. The role of corticosteroid-binding globulin in the evaluation of adrenal insufficiency. Journal of Pediatric Endocrinology and Metabolism. 2018;31(2):107-15.
8. Bhake RC, Kluckner V, Stassen H, Russell GM, Leendertz J, Stevens K, et al. Continuous Free Cortisol Profiles-Circadian Rhythms in Healthy Men. J Clin Endocrinol Metab. 2019;104(12):5935-47.
9. Timmermans S, Souffriau J, Libert C. A General Introduction to Glucocorticoid Biology. Front Immunol. 2019;10:1545.
10. Hasenmajer V, Bonaventura I, Minnetti M, Sada V, Sbardella E, Isidori AM. Non-Canonical Effects of ACTH: Insights Into Adrenal Insufficiency. Front Endocrinol (Lausanne). 2021;12:701263.
11. Hatakeyama H, Inaba S, Taniguchi N, Miyamori I. Functional adrenocorticotropic hormone receptor in cultured human vascular endothelial cells : possible role in control of blood pressure. Hypertension. 2000;36(5):862-5.
12. Catania A, Gatti S, Colombo G, Lipton JM. Targeting melanocortin receptors as a novel strategy to control inflammation. Pharmacol Rev. 2004;56(1):1-29.
13. Wang W, Guo DY, Lin YJ, Tao YX. Melanocortin Regulation of Inflammation. Front Endocrinol (Lausanne). 2019;10:683.
14. Montero-Melendez T. ACTH: The forgotten therapy. Semin Immunol. 2015;27(3):216-26.
15. Addison T. On the constitutional and local effects of disease of the supra-renal capsules. . 1855.
16. Libraries TUoID. Plates and plate descriptions from On the constitutional and local effects of disease of the supra-renal capsules, 1855.
17. Blizzard RM, Kyle MA, Chandler RW, Hung W. ADRENAL ANTIBODIES IN ADDISON'S DISEASE. The Lancet. 1962;280(7262):901-3.
18. Winqvist O, Karlsson FA, Kämpe O. 21-Hydroxylase, a major autoantigen in idiopathic Addison's disease. Lancet (London, England). 1992;339(8809):1559-62.
19. Betterle C, Presotto F, Furmaniak J. Epidemiology, pathogenesis, and diagnosis of Addison's disease in adults. J Endocrinol Invest. 2019.
20. Erichsen MM, Lovas K, Skinningsrud B, Wolff AB, Undlien DE, Svartberg J, et al. Clinical, immunological, and genetic features of autoimmune primary adrenal insufficiency: observations from a Norwegian registry. J Clin Endocrinol Metab. 2009;94(12):4882-90.

21. Dalin F, Nordling Eriksson G, Dahlqvist P, Hallgren A, Wahlberg J, Ekwall O, et al. Clinical and immunological characteristics of Autoimmune Addison's disease: a nationwide Swedish multicenter study. J Clin Endocrinol Metab. 2016:jc20162522.
22. Mason AS, Meade TW, Lee JA, Morris JN. Epidemiological and clinical picture of Addison's disease. Lancet. 1968;2(7571):744-7.
23. Olafsson AS, Sigurjonsdottir HA. INCREASING PREVALENCE OF ADDISON DISEASE: RESULTS FROM A NATIONWIDE STUDY. Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists. 2016;22(1):30-5.
24. Husebye ES, Pearce SH, Krone NP, Kämpe O. Adrenal insufficiency. Lancet (London, England). 2021;397(10274):613-29.
25. Husebye ES, Anderson MS, Kämpe O. Autoimmune Polyendocrine Syndromes. The New England journal of medicine. 2018;378(26):2543-4.
26. Betterle C, Furmaniak J, Sabbadin C, Scaroni C, Presotto F. Type 3 autoimmune polyglandular syndrome (APS-3) or type 3 multiple autoimmune syndrome (MAS-3): an expanding galaxy. Journal of endocrinological investigation. 2023;46(4):643-65.
27. Hellesen A, Bratland E, Husebye ES. Autoimmune Addison's disease - An update on pathogenesis. Ann Endocrinol (Paris). 2018;79(3):157-63.
28. Betterle C, Pra CD, Pedini B, Zanchetta R, Albergoni MP, Chen S, et al. Assessment of adrenocortical function and autoantibodies in a baby born to a mother with autoimmune polyglandular syndrome Type 2. Journal of endocrinological investigation. 2004;27(7):618-21.
29. Wolff AB, Breivik L, Hufthammer KO, Grytaas MA, Bratland E, Husebye ES, et al. The natural history of 21-hydroxylase autoantibodies in autoimmune Addison's disease. European journal of endocrinology / European Federation of Endocrine Societies. 2021;184(4):607-15.
30. Gitto L, Stoppacher R, Serinelli S. Death Due to Adrenal Crisis: Case Report and a Review of the Forensic Literature. Am J Forensic Med Pathol. 2021;42(4):392-6.
31. Irvine WJ, Stewart AG, Scarth L. A clinical and immunological study of adrenocortical insufficiency (Addison's disease). Clin Exp Immunol. 1967;2(1):31-70.
32. SAPHIR O, BINSWANGER HF. SUPRARENAL CORTICAL INSUFFICIENCY AND CYTOTOXIC CONTRACTION OF THE SUPRARENALS. Journal of the American Medical Association. 1930;95(14):1007-11.
33. HALL EM, HEMKEN L. THE ADRENAL GLANDS: A CLINICAL AND PATHOLOGIC STUDY. Archives of Internal Medicine. 1936;58(3):448-68.
34. Guttman PH. Addison's disease: a study of the pathology and a statistical analysis: University of Minnesota; 1930.
35. Eisenbarth GS, Gottlieb PA. Autoimmune polyendocrine syndromes. N Engl J Med. 2004;350(20):2068-79.
36. Naletto L, Frigo AC, Ceccato F, Sabbadin C, Scarpa R, Presotto F, et al. The natural history of autoimmune Addison's disease from the detection of autoantibodies to development of the disease: a long-term follow-up study on 143 patients. European journal of endocrinology / European Federation of Endocrine Societies. 2019;180(3):223-34.
37. Howarth S, Giovanelli L, Napier C, Pearce SH. Heterogeneous natural history of Addison's disease: mineralocorticoid deficiency may predominate. Endocr Connect. 2023;12(1).
38. Del Pilar Larosa M, Chen S, Steinmaus N, Macrae H, Guo L, Masiero S, et al. A new ELISA for autoantibodies to steroid 21-hydroxylase. Clin Chem Lab Med. 2018;56(6):933-8.
39. Torrejón S, Webb SM, Rodríguez-Espinosa J, Martínez de Osaba MJ, Corcoy R. Long-lasting subclinical Addison's disease. Exp Clin Endocrinol Diabetes. 2007;115(8):530-2.
40. Rosenthal FD, Davies MK, Burden AC. Malignant disease presenting as Addison's disease. Br Med J. 1978;1(6127):1591-2.
41. Manso J, Pezzani R, Scarpa R, Gallo N, Betterle C. The natural history of autoimmune Addison's disease with a non-classical presentation: a case report and review of literature. Clinical chemistry and laboratory medicine. 2018;56(6):896-900.

42. De Bellis A, Bizzarro A, Rossi R, Paglionico VA, Criscuolo T, Lombardi G, et al. Remission of subclinical adrenocortical failure in subjects with adrenal autoantibodies. J Clin Endocrinol Metab. 1993;76(4):1002-7.
43. Chakera AJ, Vaidya B. Spontaneously resolving Addison's disease. Qjm. 2012;105(11):1113-5.
44. Baxter M, Gorick S, Swords FM. Recovery of adrenal function in a patient with confirmed Addison's disease. Endocrinol Diabetes Metab Case Rep. 2013;2013:130070.
45. Smans LC, Zelissen PM. Partial recovery of adrenal function in a patient with autoimmune Addison's disease. J Endocrinol Invest. 2008;31(7):672-4.
46. Smans LC, Zelissen PM. Does recovery of adrenal function occur in patients with autoimmune Addison's disease? Clin Endocrinol (Oxf). 2011;74(4):434-7.
47. Vulto A, Bergthorsdottir R, van Faassen M, Kema IP, Johannsson G, van Beek AP. Residual endogenous corticosteroid production in patients with adrenal insufficiency. Clin Endocrinol (Oxf). 2019;91(3):383-90.
48. Napier C, Allinson K, Gan EH, Mitchell AL, Gilligan LC, Taylor AE, et al. Natural history of adrenal steroidogenesis in autoimmune Addison's disease following diagnosis and treatment. J Clin Endocrinol Metab. 2020.
49. Bleicken B, Hahner S, Ventz M, Quinkler M. Delayed diagnosis of adrenal insufficiency is common: a cross-sectional study in 216 patients. The American journal of the medical sciences. 2010;339(6):525-31.
50. Papierska L, Rabijewski M. Delay in diagnosis of adrenal insufficiency is a frequent cause of adrenal crisis. Int J Endocrinol. 2013;2013:482370.
51. Bornstein SR, Allolio B, Arlt W, Barthel A, Don-Wauchope A, Hammer GD, et al. Diagnosis and Treatment of Primary Adrenal Insufficiency: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab. 2016:jc20151710.
52. Husebye ES, Allolio B, Arlt W, Badenhoop K, Bensing S, Betterle C, et al. Consensus statement on the diagnosis, treatment and follow-up of patients with primary adrenal insufficiency. J Intern Med. 2014;275(2):104-15.
53. Wass JA, Arlt W. How to avoid precipitating an acute adrenal crisis. Bmj. 2012;345:e6333.
54. Ueland GA, Methlie P, Oksnes M, Thordarson HB, Sagen J, Kellmann R, et al. The Short Cosyntropin Test Revisited: New Normal Reference Range Using LC-MS/MS. J Clin Endocrinol Metab. 2018;103(4):1696-703.
55. Murray RD, Ekman B, Uddin S, Marelli C, Quinkler M, Zelissen PM. Management of glucocorticoid replacement in adrenal insufficiency shows notable heterogeneity - data from the EU-AIR. Clinical endocrinology. 2017;86(3):340-6.
56. Staufenbiel SM, Andela CD, Manenschijn L, Pereira AM, van Rossum EFC, Biermasz NR. Increased Hair Cortisol Concentrations and BMI in Patients With Pituitary-Adrenal Disease on Hydrocortisone Replacement. The Journal of Clinical Endocrinology & Metabolism. 2015;100(6):2456-62.
57. Sævik Å B, Wolff AB, Björnsdottir S, Simunkova K, Hynne MS, Dolan DWP, et al. Potential Transcriptional Biomarkers to Guide Glucocorticoid Replacement in Autoimmune Addison's Disease. J Endocr Soc. 2021;5(3):bvaa202.
58. Choudhury S, Lightman S, Meeran K. Improving glucocorticoid replacement profiles in adrenal insufficiency. Clin Endocrinol (Oxf). 2019;91(3):367-71.
59. Saverino S, Falorni A. Autoimmune Addison's disease. Best Pract Res Clin Endocrinol Metab. 2020:101379.
60. Nowotny H, Ahmed SF, Bensing S, Beun JG, Brösamle M, Chifu I, et al. Therapy options for adrenal insufficiency and recommendations for the management of adrenal crisis. Endocrine. 2021;71(3):586-94.
61. EMA. Efmody: European Union; 2022 [Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/efmody.

62. Quinkler M, Oelkers W, Remde H, Allolio B. Mineralocorticoid substitution and monitoring in primary adrenal insufficiency. Best Pract Res Clin Endocrinol Metab. 2015;29(1):17-24.
63. Burger-Stritt S, Eff A, Quinkler M, Kienitz T, Stamm B, Willenberg HS, et al. Standardised patient education in adrenal insufficiency: a prospective multi-centre evaluation. European journal of endocrinology / European Federation of Endocrine Societies. 2020;183(2):119-27.
64. Husebye ES. Binyrebarksvikt; årsak, diagnostikk og behandling. Indremedisineren. 2017.
65. Burger-Stritt S, Pulzer A, Hahner S. Quality of Life and Life Expectancy in Patients with Adrenal Insufficiency: What Is True and What Is Urban Myth? Front Horm Res. 2016;46:171-83.
66. Erichsen MM, Lovas K, Fougner KJ, Svartberg J, Hauge ER, Bollerslev J, et al. Normal overall mortality rate in Addison's disease, but young patients are at risk of premature death. Eur J Endocrinol. 2009;160(2):233-7.
67. Ueland GA, Husebye ES. Metabolic Complications in Adrenal Insufficiency. Front Horm Res. 2018;49:104-13.
68. Rushworth RL, Torpy DJ, Falhammar H. Adrenal Crisis. The New England journal of medicine. 2019;381(9):852-61.
69. Allolio B. Extensive expertise in endocrinology. Adrenal crisis. Eur J Endocrinol. 2015;172(3):R115-24.
70. Claessen K, Andela CD, Biermasz NR, Pereira AM. Clinical Unmet Needs in the Treatment of Adrenal Crisis: Importance of the Patient's Perspective. Front Endocrinol (Lausanne). 2021;12:701365.
71. Puar TH, Stikkelbroeck NM, Smans LC, Zelissen PM, Hermus AR. Adrenal Crisis: Still a Deadly Event in the 21 Century. Am J Med. 2015.
72. Hahner S, Spinnler C, Fassnacht M, Burger-Stritt S, Lang K, Milovanovic D, et al. High incidence of adrenal crisis in educated patients with chronic adrenal insufficiency: a prospective study. The Journal of clinical endocrinology and metabolism. 2015;100(2):407-16.
73. Rushworth RL, Torpy DJ, Falhammar H. Adrenal Crisis. Reply. The New England journal of medicine. 2019;381(22):2182-3.
74. Hahner S, Loeffler M, Bleicken B, Drechsler C, Milovanovic D, Fassnacht M, et al. Epidemiology of adrenal crisis in chronic adrenal insufficiency: the need for new prevention strategies. Eur J Endocrinol. 2010;162(3):597-602.
75. Smans LC, Van der Valk ES, Hermus AR, Zelissen PM. Incidence of adrenal crisis in patients with adrenal insufficiency. Clin Endocrinol (Oxf). 2016;84(1):17-22.
76. Meyer G, Neumann K, Badenhoop K, Linder R. Increasing prevalence of Addison's disease in German females: health insurance data 2008-2012. Eur J Endocrinol. 2014;170(3):367-73.
77. Quinkler M, Murray RD, Zhang P, Marelli C, Petermann R, Isidori AM, et al. Characterization of patients with adrenal insufficiency and frequent adrenal crises. European journal of endocrinology / European Federation of Endocrine Societies. 2021;184(6):761-71.
78. Bergthorsdottir R, Leonsson-Zachrisson M, Oden A, Johannsson G. Premature mortality in patients with Addison's disease: a population-based study. The Journal of clinical endocrinology and metabolism. 2006;91(12):4849-53.
79. Bensing S, Brandt L, Tabaroj F, Sjoberg O, Nilsson B, Ekbom A, et al. Increased death risk and altered cancer incidence pattern in patients with isolated or combined autoimmune primary adrenocortical insufficiency. Clin Endocrinol (Oxf). 2008;69(5):697-704.
80. Ngaosuwan K, Johnston DG, Godsland IF, Cox J, Majeed A, Quint JK, et al. Cardiovascular Disease in Patients With Primary and Secondary Adrenal Insufficiency and the Role of Comorbidities. The Journal of clinical endocrinology and metabolism. 2021;106(5):1284-93.
81. Puglisi S, Rossini A, Tabaro I, Cannavò S, Ferrau F, Ragonese M, et al. What factors have impact on glucocorticoid replacement in adrenal insufficiency: a real-life study. Journal of endocrinological investigation. 2021;44(4):865-72.
82. Skov J, Sundstrom A, Ludvigsson JF, Kampe O, Bensing S. Sex-specific risk of cardiovascular disease in autoimmune Addison's disease - a population-based cohort study. J Clin Endocrinol Metab. 2019.

83. Bergthorsdottir R, Ragnarsson O, Skrtic S, Glad CAM, Nilsson S, Ross IL, et al. Visceral Fat and Novel Biomarkers of Cardiovascular Disease in Patients With Addison's Disease: A Case-Control Study. The Journal of clinical endocrinology and metabolism. 2017;102(11):4264-72.
84. Ross IL, Bergthorsdottir R, Levitt N, Dave JA, Schatz D, Marais D, et al. Cardiovascular risk factors in patients with Addison's disease: a comparative study of South African and Swedish patients. PloS one. 2014;9(6):e90768.
85. Isidori AM, Venneri MA, Graziadio C, Simeoli C, Fiore D, Hasenmajer V, et al. Effect of once-daily, modified-release hydrocortisone versus standard glucocorticoid therapy on metabolism and innate immunity in patients with adrenal insufficiency (DREAM): a single-blind, randomised controlled trial. Lancet Diabetes Endocrinol. 2018;6(3):173-85.
86. Bjornsdottir S, Sundstrom A, Ludvigsson JF, Blomqvist P, Kampe O, Bensing S. Drug prescription patterns in patients with Addison's disease: a Swedish population-based cohort study. The Journal of clinical endocrinology and metabolism. 2013;98(5):2009-18.
87. Giordano R, Marzotti S, Balbo M, Romagnoli S, Marinazzo E, Berardelli R, et al. Metabolic and cardiovascular profile in patients with Addison's disease under conventional glucocorticoid replacement. Journal of endocrinological investigation. 2009;32(11):917-23.
88. Choudhury S, Lightman S, Meeran K. Improving glucocorticoid replacement profiles in adrenal insufficiency. Clin Endocrinol (Oxf). 2019.
89. Rahvar AH, Riesel M, Graf T, Harbeck B. Cardiovascular outcome in patients with adrenal insufficiency-a therapeutic dilemma. Endocrine. 2021;72(2):582-5.
90. Mongioì LM, Condorelli RA, Barbagallo F, La Vignera S, Calogero AE. Dual-release hydrocortisone for treatment of adrenal insufficiency: a systematic review. Endocrine. 2020;67(3):507-15.
91. Fichna M, Fichna P, Gryczyńska M, Czarnywojtek A, Żurawek M, Ruchała M. Steroid replacement in primary adrenal failure does not appear to affect circulating adipokines. Endocrine. 2015;48(2):677-85.
92. Willerson JT, Ridker PM. Inflammation as a Cardiovascular Risk Factor. Circulation. 2004;109(21_suppl_1):II-2-II-10.
93. Schakelaar MY, Kemperman H, Schoneveld AH, Hoefer IE, Tiel Groenestege WM. Analysis of C-reactive protein from finger stick dried blood spot to predict high risk of cardiovascular disease. Scientific reports. 2023;13(1):2515.
94. Conrad N, Verbeke G, Molenberghs G, Goetschalckx L, Callender T, Cambridge G, et al. Autoimmune diseases and cardiovascular risk: a population-based study on 19 autoimmune diseases and 12 cardiovascular diseases in 22 million individuals in the UK. Lancet (London, England). 2022;400(10354):733-43.
95. Agca R, Smulders Y, Nurmohamed M. Cardiovascular disease risk in immune-mediated inflammatory diseases: recommendations for clinical practice. Heart. 2022;108(1):73-9.
96. Rahvar AH, Riesel M, Graf T, Harbeck B. Adrenal insufficiency treated with conventional hydrocortisone leads to elevated levels of Interleukin-6: a pilot study. Endocrine. 2019;64(3):727-9.
97. Bratland E, Hellesen A, Husebye ES. Induction of CXCL10 chemokine in adrenocortical cells by stimulation through toll-like receptor 3. Molecular and cellular endocrinology. 2013;365(1):75-83.
98. Edvardsen K, Bjånesøy T, Hellesen A, Breivik L, Bakke M, Husebye ES, et al. Peripheral Blood Cells from Patients with Autoimmune Addison's Disease Poorly Respond to Interferons In Vitro, Despite Elevated Serum Levels of Interferon-Inducible Chemokines. J Interferon Cytokine Res. 2015;35(10):759-70.
99. Bellastella G, Rotondi M, Pane E, Costantini S, Colella C, Calemma R, et al. Simultaneous evaluation of the circulating levels of both Th1 and Th2 chemokines in patients with autoimmune Addison's disease. Journal of endocrinological investigation. 2011;34(11):831-4.
100. Ekman B, Alstrand N, Bachrach-Lindström M, Jenmalm MC, Wahlberg J. Altered chemokine Th1/Th2 balance in Addison's disease: relationship with hydrocortisone dosing and quality of life.

Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2014;46(1):48-53.
101. Bothou C, Beuschlein F, Spyroglou A. Links between aldosterone excess and metabolic complications: A comprehensive review. Diabetes Metab. 2019.
102. Iwen KA, Senyaman O, Schwartz A, Drenckhan M, Meier B, Hadaschik D, et al. Melanocortin crosstalk with adipose functions: ACTH directly induces insulin resistance, promotes a pro-inflammatory adipokine profile and stimulates UCP-1 in adipocytes. J Endocrinol. 2008;196(3):465-72.
103. Whitworth JA, Saines D, Thatcher R, Butkus A, Scoggins BA. Blood pressure and metabolic effects of ACTH in normotensive and hypertensive man. Clin Exp Hypertens A. 1983;5(4):501-22.
104. Didriksen NM, Sævik Å B, Sortland LS, Øksnes M, Husebye ES. Sex-Specific Limitations in Physical Health in Primary Adrenal Insufficiency. Front Endocrinol (Lausanne). 2021;12:718660.
105. van der Valk ES, Smans LC, Hofstetter H, Stubbe JH, de Vries M, Backx FJ, et al. Decreased physical activity, reduced QoL and presence of debilitating fatigue in patients with Addison's disease. Clin Endocrinol (Oxf). 2016;85(3):354-60.
106. Li D, Brand S, Hamidi O, Westfall AA, Suresh M, Else T, et al. Quality of Life and its Determinants in Patients With Adrenal Insufficiency: A Survey Study at 3 Centers in the United States. J Clin Endocrinol Metab. 2022;107(7):e2851-e61.
107. Oksnes M, Bjornsdottir S, Isaksson M, Methlie P, Carlsen S, Nilsen RM, et al. Continuous subcutaneous hydrocortisone infusion versus oral hydrocortisone replacement for treatment of addison's disease: a randomized clinical trial. The Journal of clinical endocrinology and metabolism. 2014;99(5):1665-74.
108. Gagliardi L, Nenke MA, Thynne TR, von der Borch J, Rankin WA, Henley DE, et al. Continuous subcutaneous hydrocortisone infusion therapy in Addison's disease: a randomized, placebo-controlled clinical trial. The Journal of clinical endocrinology and metabolism. 2014;99(11):4149-57.
109. Meyer G, Koch M, Herrmann E, Bojunga J, Badenhoop K. Longitudinal AddiQoL scores may identify higher risk for adrenal crises in Addison's disease. Endocrine. 2018;60(2):355-61.
110. FDA-NIH Biomarker Working Group. BEST (Biomarkers E, and other Tools) Resource [Internet]. Silver, Spring (MD): Food and Drug Administration (US); 2016-. Co-published by National Institutes of Health (US) BM.
111. FDA. Clinical Outcome Assessments (COAs) in Medical Device Decision Making 2022 [Available from: https://www.fda.gov/about-fda/cdrh-patient-science-and-engagement-program/clinical-outcome-assessments-coas-medical-device-decision-making.
112. Rushworth RL, Torpy DJ, Falhammar H. Adrenal crises: perspectives and research directions. Endocrine. 2017;55(2):336-45.
113. Methlie P, Hustad SS, Kellmann R, Almas B, Erichsen MM, Husebye E, et al. Multisteroid LC-MS/MS assay for glucocorticoids and androgens, and its application in Addison's disease. Endocrine connections. 2013;2(3):125-36.
114. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. Nucleic Acids Res. 2011;39(15):e102.
115. Assarsson E, Lundberg M, Holmquist G, Björkesten J, Thorsen SB, Ekman D, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PloS one. 2014;9(4):e95192.
116. Olink. How is the Limit of Detection (LOD) estimated and how is this handled in the data analysis 2018 [Available from: https://olink.com/faq/how-is-the-limit-of-detection-lod-estimated-and-handled/.
117. Gan EH, MacArthur K, Mitchell AL, Hughes BA, Perros P, Ball SG, et al. Residual adrenal function in autoimmune Addison's disease: improvement after tetracosactide (ACTH1-24) treatment. The Journal of clinical endocrinology and metabolism. 2014;99(1):111-8.
118. Mohan S, Gu S, Parikh A, Radhakrishnan J. Prevalence of hyponatremia and association with mortality: results from NHANES. Am J Med. 2013;126(12):1127-37.e1.

119. Liamis G, Filippatos TD, Liontos A, Elisaf MS. MANAGEMENT OF ENDOCRINE DISEASE: Hypothyroidism-associated hyponatremia: mechanisms, implications and treatment. European journal of endocrinology / European Federation of Endocrine Societies. 2017;176(1):R15-r20.
120. Wolf P, Beiglböck H, Smaijs S, Wrba T, Rasoul-Rockenschaub S, Marculescu R, et al. Hypothyroidism and Hyponatremia: Rather Coincidence Than Causality. Thyroid. 2017;27(5):611-5.
121. Murray JS, Jayarajasingh R, Perros P. Deterioration of symptoms after start of thyroid hormone replacement. BMJ : British Medical Journal. 2001;323(7308):332-3.
122. Pearce SHS, Gan EH, Napier C. MANAGEMENT OF ENDOCRINE DISEASE: Residual adrenal function in Addison's disease. European journal of endocrinology / European Federation of Endocrine Societies. 2021;184(2):R61-r7.
123. Tarçın G, Ercan O. Emergence of Ectopic Adrenal Tissues-What are the Probable Mechanisms? J Clin Res Pediatr Endocrinol. 2022;14(3):258-66.
124. Dumontet T, Sahut-Barnola I, Septier A, Montanier N, Plotton I, Roucher-Boulez F, et al. PKA signaling drives reticularis differentiation and sexually dimorphic adrenal cortex renewal. JCI Insight. 2018;3(2).
125. Grabek A, Dolfi B, Klein B, Jian-Motamedi F, Chaboissier MC, Schedl A. The Adult Adrenal Cortex Undergoes Rapid Tissue Renewal in a Sex-Specific Manner. Cell Stem Cell. 2019;25(2):290-6.e2.
126. Lyraki R, Grabek A, Tison A, Weerasinghe Arachchige LC, Peitzsch M, Bechmann N, et al. Crosstalk between androgen receptor and WNT/β-catenin signaling causes sex-specific adrenocortical hyperplasia in mice. Dis Model Mech. 2023;16(6).
127. Lyraki R, Schedl A. The Sexually Dimorphic Adrenal Cortex: Implications for Adrenal Disease. Int J Mol Sci. 2021;22(9).
128. Prete A, Subramanian A, Bancos I, Chortis V, Tsagarakis S, Lang K, et al. Cardiometabolic Disease Burden and Steroid Excretion in Benign Adrenal Tumors : A Cross-Sectional Multicenter Study. Ann Intern Med. 2022;175(3):325-34.
129. Nanba K, Rainey WE. GENETICS IN ENDOCRINOLOGY: Impact of race and sex on genetic causes of aldosterone-producing adenomas. European journal of endocrinology / European Federation of Endocrine Societies. 2021;185(1):R1-r11.
130. Klein SL, Flanagan KL. Sex differences in immune responses. Nature Reviews Immunology. 2016;16(10):626-38.
131. Kalinowska A, Orlinska B, Panasiuk M, Jamiolkowska M, Zasim A, Florys B, et al. Assessment of preservation of beta-cell function in children with long-standing type 1 diabetes with "ultrasensitive c-peptide" method. Pediatr Endocrinol Diabetes Metab. 2017;23(3):130-8.
132. McKeigue PM, Spiliopoulou A, McGurnaghan S, Colombo M, Blackbourn L, McDonald TJ, et al. Persistent C-peptide secretion in Type 1 diabetes and its relationship to the genetic architecture of diabetes. BMC Medicine. 2019;17(1):165.
133. Pearce SH, Mitchell AL, Bennett S, King P, Chandran S, Nag S, et al. Adrenal steroidogenesis after B lymphocyte depletion therapy in new-onset Addison's disease. J Clin Endocrinol Metab. 2012;97(10):E1927-32.
134. Napier C, Gan EH, Mitchell AL, Gilligan LC, Rees DA, Moran C, et al. Residual Adrenal Function in Autoimmune Addison's Disease - Effect of Dual Therapy with Rituximab and Depot Tetracosactide. J Clin Endocrinol Metab. 2019.
135. Sonar S, Lal G. Role of Tumor Necrosis Factor Superfamily in Neuroinflammation and Autoimmunity. Front Immunol. 2015;6:364.
136. Kashio Y, Nakamura K, Abedin MJ, Seki M, Nishi N, Yoshida N, et al. Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway. J Immunol. 2003;170(7):3631-6.
137. Lei Y, Takahama Y. XCL1 and XCR1 in the immune system. Microbes Infect. 2012;14(3):262-7.
138. Wehler TC, Karg M, Distler E, Konur A, Nonn M, Meyer RG, et al. Rapid identification and sorting of viable virus-reactive CD4(+) and CD8(+) T cells based on antigen-triggered CD137 expression. J Immunol Methods. 2008;339(1):23-37.

139. van Wanrooij RL, Zwiers A, Kraal G, Bouma G. Genetic variations in interleukin-12 related genes in immune-mediated diseases. J Autoimmun. 2012;39(4):359-68.
140. Choy EH, De Benedetti F, Takeuchi T, Hashizume M, John MR, Kishimoto T. Translating IL-6 biology into effective treatments. Nat Rev Rheumatol. 2020;16(6):335-45.
141. Bianconi V, Sahebkar A, Atkin SL, Pirro M. The regulation and importance of monocyte chemoattractant protein-1. Curr Opin Hematol. 2018;25(1):44-51.
142. Dong H, Zhang Y, Huang Y, Deng H. Pathophysiology of RAGE in inflammatory diseases. Front Immunol. 2022;13:931473.
143. Robling AG, Bonewald LF. The Osteocyte: New Insights. Annu Rev Physiol. 2020;82:485-506.
144. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun. 1993;192(2):553-60.
145. Lakhani I, Gong M, Wong WT, Bazoukis G, Lampropoulos K, Wong SH, et al. Fibroblast growth factor 21 in cardio-metabolic disorders: a systematic review and meta-analysis. Metabolism. 2018;83:11-7.
146. Lee J-T, Pamir N, Liu N-C, Kirk EA, Averill MM, Becker L, et al. Macrophage Metalloelastase (MMP12) Regulates Adipose Tissue Expansion, Insulin Sensitivity, and Expression of Inducible Nitric Oxide Synthase. Endocrinology. 2014;155(9):3409-20.
147. Tinoco R, Otero DC, Takahashi AA, Bradley LM. PSGL-1: A New Player in the Immune Checkpoint Landscape. Trends Immunol. 2017;38(5):323-35.
148. Consortium TU. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Research. 2022:spondin 2 homo sapiens.
149. Mueller JW, Gilligan LC, Idkowiak J, Arlt W, Foster PA. The Regulation of Steroid Action by Sulfation and Desulfation. Endocrine reviews. 2015;36(5):526-63.
150. Geng L, Lam KSL, Xu A. The therapeutic potential of FGF21 in metabolic diseases: from bench to clinic. Nature reviews Endocrinology. 2020;16(11):654-67.
151. Díaz-catalán D V-BA, Mora M., Rodrigo M, Boswell L, Casals G, and Hanzu F. A. Effects of Fibroblast factor 21 to adrenal renewal after chronic hypercortisolism. Endocrine Abstracts (2022) 83 AO4 2022.
152. Sarwar N, Butterworth AS, Freitag DF, Gregson J, Willeit P, Gorman DN, et al. Interleukin-6 receptor pathways in coronary heart disease: a collaborative meta-analysis of 82 studies. Lancet (London, England). 2012;379(9822):1205-13.
153. Bacchiega BC, Bacchiega AB, Usnayo MJ, Bedirian R, Singh G, Pinheiro GD. Interleukin 6 Inhibition and Coronary Artery Disease in a High-Risk Population: A Prospective Community-Based Clinical Study. J Am Heart Assoc. 2017;6(3).
154. Dencker M, Björgell O, Hlebowicz J. Effect of food intake on 92 neurological biomarkers in plasma. Brain Behav. 2017;7(9):e00747.
155. Regitz-Zagrosek V, Kararigas G. Mechanistic Pathways of Sex Differences in Cardiovascular Disease. Physiological Reviews. 2017;97(1):1-37.
156. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience. 2008;9(1):46-56.
157. LaFleur MW, Muroyama Y, Drake CG, Sharpe AH. Inhibitors of the PD-1 Pathway in Tumor Therapy. J Immunol. 2018;200(2):375-83.
158. Mitchell AL, Cordell HJ, Soemedi R, Owen K, Skinningsrud B, Wolff AB, et al. Programmed Death Ligand 1 (PD-L1) Gene Variants Contribute to Autoimmune Addison's Disease and Graves' Disease Susceptibility. The Journal of Clinical Endocrinology & Metabolism. 2009;94(12):5139-45.
159. Angelousi A, Margioris AN, Tsatsanis C. ACTH Action on the Adrenals. In: Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., editors. Endotext. South Dartmouth (MA): MDText.com, Inc.

160. Nasjonalt register for organspesifikke autoimmune

sykdommer. Dekningsgradsanalyse. https://helse-bergen.no/seksjon/ROAS/Documents/Dekningsgrad%20rapport%20ROAS%20jan%202020.pdf: Helsedirektoratet. Nasjonal tjeneste for validering og

dekningsgradsanalyser.; 2020.
161. Napier C, Gan EH, Mitchell AL, Gilligan LC, Rees DA, Moran C, et al. Residual Adrenal Function in Autoimmune Addison's Disease-Effect of Dual Therapy With Rituximab and Depot Tetracosactide. J Clin Endocrinol Metab. 2020;105(4):e1250-9.
162. Schiffer L, Barnard L, Baranowski ES, Gilligan LC, Taylor AE, Arlt W, et al. Human steroid biosynthesis, metabolism and excretion are differentially reflected by serum and urine steroid metabolomes: A comprehensive review. J Steroid Biochem Mol Biol. 2019;194:105439.
163. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr Rev. 2011;32(1):81-151.
164. William A. Jeffers FACP, Harold A. Zintel, Joseph H. Hafkenschiel, A. Gorman Hills, Alfred M. Sellers, Charles C. Wolferth, F.A.C.P. The Clinical Course, Following Adrenal Resection and Sympathectomy, of 82 Patients with Severe Hypertension. Annals of Internal Medicine. 1953;39(2):254-66.
165. Soule S, Van Zyl Smit C, Parolis G, Attenborough S, Peter D, Kinvig S, et al. The low dose ACTH stimulation test is less sensitive than the overnight metyrapone test for the diagnosis of secondary hypoadrenalism. Clinical endocrinology. 2000;53(2):221-7.
166. Palstrøm NB, Matthiesen R, Rasmussen LM, Beck HC. Recent Developments in Clinical Plasma Proteomics—Applied to Cardiovascular Research. Biomedicines. 2022;10(1):162.
167. Hueber W, Robinson WH. Genomics and proteomics: Applications in autoimmune diseases. Pharmgenomics Pers Med. 2009;2:39-48.
168. Wik L, Nordberg N, Broberg J, Björkesten J, Assarsson E, Henriksson S, et al. Proximity Extension Assay in Combination with Next-Generation Sequencing for High-throughput Proteome-wide Analysis. Mol Cell Proteomics. 2021;20:100168.
169. Menyhart O, Weltz B, Győrffy B. MultipleTesting.com: A tool for life science researchers for multiple hypothesis testing correction. PloS one. 2021;16(6):e0245824.
170. Eriksson D, Røyrvik EC, Aranda-Guillén M, Berger AH, Landegren N, Artaza H, et al. GWAS for autoimmune Addison's disease identifies multiple risk loci and highlights AIRE in disease susceptibility. Nat Commun. 2021;12(1):959.
171. Silins I, Sundin A, Lubberink M, O'Sullivan L, Gurnell M, Aigbirhio F, et al. First-in-human evaluation of [(18)F]CETO: a novel tracer for adrenocortical tumours. Eur J Nucl Med Mol Imaging. 2023;50(2):398-409.
172. Hays RD, Sherbourne CD, Mazel RM. The RAND 36-Item Health Survey 1.0. Health Econ. 1993;2(3):217-27.
173. Oksnes M, Bensing S, Hulting AL, Kampe O, Hackemann A, Meyer G, et al. Quality of life in European patients with Addison's disease: validity of the disease-specific questionnaire AddiQoL. The Journal of clinical endocrinology and metabolism. 2012;97(2):568-76.

9. Appendix

9.1 Structured History Form in Paper II (in English)

Background	
Etiology	
Year of diagnosis	
Informed consent	
Sex	
Diagnostic criteria	
Elevated ACTH, low cortisol	
Pathological cosyntropin test	
Chronic use of (hydro)cortisone and fludrocortisone	
Positive for 21-OH-autoantibodies (at least once)	
Autoimmunity	
Diabetes mellitus type 1	
Year of diagnosis	
Autoimmune hypothyroidism	

Year of diagnosis	
Autoimmune hyperthyroidism	
Year of diagnosis	
Hypoparathyroidism	
Year of diagnosis	
Celiac disease	
Year of diagnosis	
Vitamin B12 deficiency	
Year of diagnosis	
Autoimmune atrophic gastritis	
Year of diagnosis	
Primary testicular insufficiency	
Year of diagnosis	
Primary ovarian insufficiency	
Year of diagnosis)	
Vitiligo	
Year of diagnosis	
Alopecia	
Year of diagnosis	
Chronic mucocutaneous candidiasis	

Year of diagnosis	
Hypophysitis	
Year of diagnosis	
Autoimmune polyendocrine syndrome	
Family history of autoimmune disease	
Treatment	
Cortisone acetate/ hydrocortisone (mg/24h, mg+mg+mg+mg)	
Fludrocortisone (mg/24h, mg+mg+mg+mg)	
DHEA (mg/24h, mg+mg+mg+mg)	
Estrogen or oral contraceptive pills	
Selective serotonin reuptake inhibitor (SSRI)	
Other medications	
Adrenal crisis	
History of adrenal crisis	

(admitted to hospital, intravenous hydrocortisone and fluid therapy given)	
Adrenal crisis at diagnosis (admitted to hospital, intravenous hydrocortisone, and fluid therapy given)	
No. extra doses taken the last week	
No. adrenal crisis the previous year (admitted to hospital, intravenous hydrocortisone, and fluid therapy given)	
Cause(s) of adrenal crisis/ crises	
No. infections the previous year	
Type of infection(s) specified	
Steroid emergency card provided	
Emergency kit provided	
Training in intramuscular emergency injection given	
Comorbidity	
Cardiovascular disease	
Year of diagnosis of cardiovascular disease	
Osteoporosis	

Other chronic disease	
Symptoms *Answer yes if the symptom is present at least half of 3 days a week*	
Salt hunger	
Orthostatic hypotension	
Fatigue	
Anorexia	
Gastrointestinal symptoms	
Muscle / joint pain	
Sleep disturbances	
Nausea	
Clinical findings	
Height (cm)	
Weight (kg)	
BMI (kg/m^2)	
Increased pigmentation	
Systolic blood pressure sitting (mmHg)	

Diastolic blood pressure sitting (mmHg)	
Social history	
Occupation	
Highest level of education completed	
Paid work	
Social security	
Household size (no. people)	
Marital status	
No. children	
Supplementary information	

9.2 Instructions for Baseline Sampling in Paper II (in English)

Stage 1: Screening

This document includes

i) An overview of the tests to be analyzed at the local laboratory, and
ii) Instructions for sampling, handling, and transport of blood and hair samples to be analyzed at Haukeland University Hospital.

Overview of routine tests to be analyzed at the local laboratory

Laboratory test	**Material**
Hemoglobin	EDTA blood

HbA1c	EDTA blood
S-TSH	Serum
S-FT4	Serum
S-Cobalamine	Serum
S-Ferritin	Serum
S-Cobalamine	Serum
S-Sodium	Serum
S-Potassium	Serum
S-Cholesterol	Serum
S-HDL-cholesterole	Serum
S-LDL-cholesterol	Serum
S-Triglycerides	Serum
S-TPO-autoantibodies	Serum
P-Renin concentration	EDTA plasma
P-ACTH	EDTA plasma

Samples are to be sent to Haukeland for analysis

Instructions for sampling, handling, and transport of samples to be analyzed at Haukeland University Hospital.

A. Blood samples

- Necessary equipment:

- **2x 5 ml serum-separator tubes**

- **2x screw cap micro tubes (e.g. Sarstedt)**

- **1x leakage-proof transport container**

- Sampling and handling

  Any problems during the blood sampling are to be noted in the CRF for screening.

  1. Please label the two microtubes (please see the preferred labeling below)
  2. Blood samples are to be collected in 2x 5 ml serum-separator tubes.
  3. Please turn the tubes carefully 5 to 10 times.
  4. Place the tubes in a test tube rack for coagulation at room temperature for 30 to 100 minutes.
  5. Next, centrifuge the samples. Please make sure the speed is set to meet the recommendations of the producer.

6. After centrifugation is completed, please make sure that the serum separator gel constitutes a compact barrier between the blood cells and the serum.
7. Next, use a pipette to transfer the serum to the pre-labeled microtubes.
8. The microtubes should be stored at minus 80 °C or sent to Haukeland University Hospital on the same day.

9.3 HRQoL Questionnaires (in Norwegian)

RAND-36

RAND-36 Din helse

Spørsmålene under handler om hvordan du oppfatter helsen din. Disse opplysningene vil hjelpe oss til å forstå hvordan du føler deg og hvor godt du er i stand til å utføre dine vanlige aktiviteter.

Hvert spørsmål skal besvares ved å sette et kryss (X) i den boksen som passer best for deg.

1. Stort sett, vil du si at helsen din er:

Utmerket	Veldig god	God	Nokså god	Dårlig
☐	☐	☐	☐	☐

2. Sammenlignet med for ett år siden, hvordan vil du si at helsen din stort sett er nå?

Mye bedre nå enn for ett år siden	Litt bedre nå enn for ett år siden	Omtrent som for ett år siden	Litt dårligere nå enn for ett år siden	Mye dårligere nå enn for ett år siden
☐	☐	☐	☐	☐

3. De neste spørsmålene handler om aktiviteter som du kanskje utfører i løpet av en vanlig dag. Er helsen din slik at den begrenser deg i utførelsen av disse aktivitetene nå? Hvis ja, hvor mye? [Kryss (X) en boks på hver linje.]

		Ja, begrenser meg mye	Ja, begrenser meg litt	Nei, begrenser meg ikke i det hele tatt
a	**Anstrengende aktiviteter** som å løpe, løfte tunge gjenstander, delta i anstrengende idrett	☐	☐	☐
b	**Moderate aktiviteter** som å flytte et bord, støvsuge, gå en spasertur eller drive med hagearbeid	☐	☐	☐
c	Løfte eller bære poser med dagligvarer	☐	☐	☐
d	Gå opp trappen **flere** etasjer	☐	☐	☐
e	Gå opp trappen **én** etasje	☐	☐	☐
f	Bøye deg eller gå ned på kne	☐	☐	☐
g	Gå **mer enn to kilometer**	☐	☐	☐
h	Gå **flere hundre meter**	☐	☐	☐
i	Gå **hundre meter**	☐	☐	☐
j	Dusje eller kle på deg	☐	☐	☐

4. **I løpet av de siste fire ukene, har du hatt noen av de følgende problemene i arbeidet ditt eller i andre daglige aktiviteter på grunn av din fysiske helse?**

		Ja	Nei
a	Kuttet ned på **hvor mye tid** du brukte på arbeid eller andre aktiviteter	☐	☐
b	**Fått gjort mindre** enn du ønsket	☐	☐
c	Vært begrenset i type **arbeidsoppgaver** eller andre aktiviteter	☐	☐
d	Hatt **problemer** med å utføre arbeidet eller andre aktiviteter (for eksempel at det krevde en ekstra innsats av deg)	☐	☐

5. **I løpet av de siste fire ukene, har du hatt noen av de følgende problemene i arbeidet ditt eller i andre daglige aktiviteter på grunn av følelsesmessige problemer (som å føle seg engstelig eller deprimert)?**

		Ja	Nei
a	Kuttet ned på **hvor mye tid** du brukte på arbeid eller andre aktiviteter	☐	☐
b	**Fått gjort mindre** enn du ønsket	☐	☐
c	Utført arbeid eller andre aktiviteter **mindre grundig** enn vanlig	☐	☐

6. **I løpet av de siste fire ukene, i hvilken grad har den fysiske helsen din eller følelsesmessige problemer påvirket dine vanlige sosiale aktiviteter med familie, venner, naboer eller andre grupper mennesker?**

Ikke i det hele tatt	Litt	Moderat	Ganske mye	Ekstremt mye
☐	☐	☐	☐	☐

7. **Hvor mye kroppslige smerter har du hatt i løpet av de siste fire ukene?**

Ingen	Veldig svake	Svake	Moderate	Sterke	Veldig sterke
☐	☐	☐	☐	☐	☐

8. **I løpet av de siste fire ukene, hvor mye har smerter påvirket det vanlige arbeidet ditt (gjelder både arbeid utenfor hjemmet og husarbeid)?**

Ikke i det hele tatt	Litt	Moderat	Ganske mye	Ekstremt mye
☐	☐	☐	☐	☐

9. **De neste spørsmålene handler om hvordan du føler deg og hvordan du har hatt det i løpet av de siste fire ukene. For hvert spørsmål, ber vi deg velge det svaret som best beskriver hvordan du har følt deg.**

Hvor ofte i løpet av de siste fire ukene:

		Hele tiden	Mesteparten av tiden	En god del av tiden	Noe av tiden	Litt av tiden	Aldri
a	Har du følt deg full av liv?	☐	☐	☐	☐	☐	☐
b	Har du vært veldig nervøs?	☐	☐	☐	☐	☐	☐
c	Har du følt deg så langt nede at ingenting kunne gjøre deg glad?	☐	☐	☐	☐	☐	☐
d	Har du følt deg rolig og avslappet?	☐	☐	☐	☐	☐	☐
e	Har du hatt mye overskudd?	☐	☐	☐	☐	☐	☐
f	Har du følt deg nedfor og deprimert?	☐	☐	☐	☐	☐	☐
g	Har du følt deg utslitt	☐	☐	☐	☐	☐	☐
h	Har du følt deg glad?	☐	☐	☐	☐	☐	☐
i	Har du følt deg sliten?	☐	☐	☐	☐	☐	☐

10. **I løpet av de siste fire ukene, hvor mye av tiden har den fysiske helsen din eller følelsesmessige problemer påvirket dine sosiale aktiviteter (som å besøke venner, slektninger osv.)?**

Hele tiden	Mesteparten av tiden	En del av tiden	Litt av tiden	Aldri
☐	☐	☐	☐	☐

11. Hvor RIKTIG eller GAL er hver av de følgende påstandene for deg?

		Helt riktig	Stort sett riktig	Vet ikke	Stort sett galt	Helt galt
a	Det virker som om jeg blir syk litt lettere enn andre	☐	☐	☐	☐	☐
b	Jeg er like frisk som de fleste jeg kjenner	☐	☐	☐	☐	☐
c	Jeg regner med at helsen min blir dårligere	☐	☐	☐	☐	☐
d	Helsen min er utmerket	☐	☐	☐	☐	☐

AddiQoL-30

AddiQoL

Helserelatert livskvalitet ved Addisons sykdom

Spørsmålene under belyser ditt syn på helsetilstanden din de siste 4 ukene, og hva du tenker om livet generelt. Ikke bruk for lang tid på å svare, da det har vist seg at det første svaret oftest er det mest nøyaktige. Vi ber deg om å besvare alle spørsmålene.

	Ikke i det hele tatt	Litt av tiden	En del av tiden	Mye av tiden	Nesten hele tiden	Alltid
Jeg er fornøyd med min helse						
Jeg kan holde det gående hele dagen uten å bli trøtt						
Dagligdagse aktiviteter gjør meg sliten						
Jeg må ta meg sammen for å gjøre ferdig det jeg holder på med						
Jeg må presse meg selv til å gjøre ting						
Jeg mister tråden i hva jeg vil si						
Jeg sover godt						
Jeg føler meg uthvilt når jeg våkner om morgenen						
Jeg føler meg uvel når jeg våkner om morgenen						
Jeg er fornøyd med mitt seksualliv						
Jeg er avslappet						
Jeg føler meg nedstemt eller deprimert						
Jeg er irritabel						
Jeg synes det er vanskelig å tenke klart						
Jeg føler meg svimmel og ør						
Jeg svetter uten spesiell grunn						

AddiQoL

Helserelatert livskvalitet ved Addisons sykdom

	Ikke i det hele tatt	Litt av tiden	En del av tiden	Mye av tiden	Nesten hele tiden	Alltid
Jeg har hodepine						
Jeg er kvalm						
Jeg har vondt i ledd og/eller muskler						
Jeg har vondt i ryggen						
Jeg føler meg svak i beina						
Jeg er bekymret for min helsetilstand						
Min arbeidskapasitet er begrenset						
Jeg klarer godt å konsentrere meg						
Jeg føler meg glad						
Jeg føler meg full av tiltakslyst						

	Svært uenig	Uenig	Litt uenig	Litt enig	Enig	Svært enig
Jeg føler meg i god fysisk form						
Jeg blir lettere syk enn andre						
Jeg trenger lang tid på å komme meg når jeg har vært syk						
Jeg mestrer godt følelsesladede situasjoner						

9.4 Calculations of HRQoL Scores

RAND-36

The Research and Development 36-item (RAND-36) consists of 36 items grouped into one of the following eight health concepts (172): physical functioning (PF), role limitations caused by physical health problems (RP), role limitations caused by emotional problems (RE), social functioning (SF), general mental health(MH), vitality (VT), bodily pain (BP), and general health (GH). Analyzing RAND-36 scores is a four-step process. First, for each separate item, the patient marks a number between one and two, one and four, or one and six. Second, 10 items require recoding to a positive statement for higher scores to indicate better health. Third, items belonging to the same health concept are added together to create an algebraic sum. Finally, the algebraic sums are transformed into a score between 0 and 100, where a higher score represents a more favorable health state (172).

AddiQoL

AddiQoL is a disease-specific questionnaire developed by our group to assess HRQoL in patients with Addison's disease (173). The questionnaire exists in two versions, containing either eight (AddiQoL-8) or 30 (AddiQoL-30) items. For every item, patients mark their answers on a scale from one to six. Each question belongs to one of four domains: fatigue, emotional well-being, adrenal insufficiency-related symptoms, and miscellaneous. For positive statements, one represents the worst, and six represents the most favorable health state. Negative statement scores are later inversed to align with positive statements. Finally, all numbers are added to give a total score between 30 to 120 (173).

JIM Original Article

doi: 10.1111/joim.12699

Clues for early detection of autoimmune Addison's disease – myths and realities

Å. B. Sævik[1,*], A.-K. Åkerman[2,3,*], K. Grønning[4], I. Nermoen[4,5], S. F. Valland[6], T. E. Finnes[6], M. Isaksson[7], P. Dahlqvist[8], R. Bergthorsdottir[9,10], O. Ekwall[11,12], J. Skov[3,13], B. G. Nedrebø[1,14], A.-L. Hulting[3], J. Wahlberg[15], J. Svartberg[16,17], C. Höybye[3,18], I. H. Bleskestad[19], A. P. Jørgensen[20], O. Kämpe[18,21,22], M. Øksnes[1,21,23], S. Bensing[3,18,**] & E. S. Husebye[1,21,22,23,**]

From the [1]Department of Clinical Medicine, University of Bergen, Bergen, Norway; [2]Department of Medicine, Örebro University Hospital, Örebro; [3]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; [4]Division of Medicine, Akershus University Hospital; [5]Institute of Clinical Medicine, Akershus University Hospital, University of Oslo, Lørenskog; [6]Division of Endocrinology, Innlandet Hospital Trust, Hamar, Norway; [7]Department of Medical Sciences, Uppsala University, Uppsala; [8]Department of Public Health and Clinical Medicine, Umeå University, Umeå; [9]Department of Endocrinology, Sahlgrenska University Hospital; [10]Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy; [11]Department of Pediatrics, Institute of Clinical Sciences; [12]Department of Rheumatology and Inflammation Research, Institute of Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg; [13]Endocrine Division, Department of Medicine, Karlstad City Hospital, Karlstad, Sweden; [14]Department of Medicine, Haugesund Hospital, Haugesund, Norway; [15]Division of Endocrinology, Department of Medical and Health Sciences, Faculty of Health Sciences, Linköping University, Linköping, Sweden; [16]Division of Internal Medicine, University Hospital of North Norway; [17]Tromsø Endocrine Research Group, Department of Clinical Medicine, UiT The Arctic University of Norway, Tromsø, Norway; [18]Department of Endocrinology, Metabolism and Diabetes, Karolinska University Hospital, Stockholm, Sweden; [19]Department of Internal Medicine, Stavanger University Hospital, Stavanger; [20]Department of Endocrinology, Oslo University Hospital, Oslo, Norway; [21]Department of Medicine, Solna, Karolinska Institutet, Stockholm, Sweden; [22]K.G. Jebsen center for Autoimmune Disorders, University of Bergen; and [23]Department of Medicine, Haukeland University Hospital, Bergen, Norway

Abstract. Bjorvatn Sævik A, Åkerman AK, Grønning K, Nermoen I, Valland SF, Finnes TE, Isaksson M, Dahlqvist P, Bergthorsdottir R, Ekwall O, Skov J, Nedrebø BG, Hulting AL, Wahlberg J, Svartberg J, Höybye C, Bleskestad IH, Jørgensen AP, Kämpe O, Øksnes M, Bensing S, Husebye ES (University of Bergen, Norway; Örebro University Hospital, Örebro; Karolinska Institutet, Stockholm, Sweden; Akershus University Hospital; Akershus University Hospital, University of Oslo, Lørenskog; Innlandet Hospital Trust, Hamar, Norway; Uppsala University, Uppsala; Umeå University, Umeå; Sahlgrenska University Hospital and Sahlgrenska Academy, University of Gothenburg; The Sahlgrenska Academy, University of Gothenburg; The Sahlgrenska Acad emy, University of Gothenburg, Gothen burg; Karlstad City Hospital, Sweden; Haugesund Hospital, Haugesund, Norway; Linköping University, Linköping, Sweden; University Hospital of North Norway; UiT The Arctic University of Norway, Tromsø, Norway; Karolinska University Hospital, Stockholm, Sweden; Stavanger University Hospital, Stavanger; Oslo University Hospital, Oslo, Norway; Karolinska Institutet, Stockholm, Sweden; University of Bergen; Haukeland University Hospital, Bergen, Norway). Clues for early detection of autoimmune Addison's disease – myths and realities. *J Intern Med* 2018; **283**: 190–199.

Background. Early detection of autoimmune Addison's disease (AAD) is important as delay in diagnosis may result in a life-threatening adrenal crisis and death. The classical clinical picture of untreated AAD is well-described, but methodical investigations are scarce.

Objective. Perform a retrospective audit of patient records with the aim of identifying biochemical markers for early diagnosis of AAD.

Material and Methods. A multicentre retrospective study including 272 patients diagnosed with AAD at hospitals in Norway and Sweden during 1978–2016. Scrutiny of medical records provided patient data and laboratory values.

Results. Low sodium occurred in 207 of 247 (84%), but only one-third had elevated potassium. Other common nonendocrine tests were largely normal. TSH was elevated in 79 of 153 patients, and hypoglycaemia was found in 10%. Thirty-three per cent were diagnosed subsequent to adrenal crisis, in whom electrolyte disturbances were significantly more pronounced ($P < 0.001$). Serum cortisol was consistently decreased (median 62 nmol L^{-1} [1–668]) and significantly lower in individuals with adrenal crisis (38 nmol L^{-1} [2–442]) than in those without (81 nmol L^{-1} [1–668], $P < 0.001$).

Conclusion. The most consistent biochemical finding of untreated AAD was low sodium independent of

*,**These authors contributed equally to the study.

the degree of glucocorticoid deficiency. Half of the patients had elevated TSH levels. Only a minority presented with marked hyperkalaemia or other nonhormonal abnormalities. Thus, unexplained low sodium and/or elevated TSH should prompt consideration of an undiagnosed AAD, and on clinical suspicion bring about assay of cortisol and ACTH. Presence of 21-hydroxylase autoantibodies confirms autoimmune aetiology. Anticipating additional abnormalities in routine blood tests may delay diagnosis.

Keywords: Addison, adrenal insufficiency, autoimmune disease, cortisol, electrolytes, endocrinology.

Introduction

Primary adrenal insufficiency, or Addison's disease (AD), is a rare endocrine disease occurring in 100–220 per million [1]. Autoimmune destruction of the adrenal cortex accounts for 80–90% of AD cases in developed countries [2], and risk genes pertaining to the adaptive immune system have been identified [3].

Detection at an early stage is important as delay in proper treatment may be fatal. Indeed, an undiagnosed and untreated AD is lethal [4, 5]. Alarmingly, up to half of the patients develop adrenal crisis before being diagnosed [6]. To avoid deadly outcome, it is crucial that physicians are able to recognize key symptoms and signs of adrenal crisis and know how to initiate treatment immediately upon clinical suspicion.

Autoimmune Addison's disease (AAD) typically presents gradually with unspecific symptoms as fatigue, weight loss, nausea and postural dizziness. These ambiguous features pose a major challenge to early detection. As a result, diagnosis is often missed and patients frequently receive other incorrect diagnoses and treatments [7, 8]. The fact that most patients have seen multiple doctors before the correct diagnosis is suspected suggests that appropriate hormone testing was not performed and emphasizes the need for new strategies for early identification [7].

Even if the classical picture of untreated AAD is well-described, it has rarely been subject to methodical review [9, 10]. Low sodium in combination with hyperkalaemia is considered strong indicators of AAD [6, 11, 12]. Other reported features include hypercalcaemia, mild normocytic anaemia, mild eosinophilia, lymphocytosis and increased creatinine [4, 11, 13]. Hypoglycaemia may be present, although said to be more frequent in children than in adults [14–16]. Once suspected, AAD is usually easy to diagnose by measuring a paired morning cortisol and adrenocorticotropic hormone (ACTH), ideally supplemented with assay of 21-hydroxylase autoantibodies (21OH-Ab), an early and specific biomarker for AAD [17, 18].

Given the ambiguous presentation, and the fact that when eventually diagnosed, many are in adrenal crisis [19], we asked whether there are reliable clues in commonly taken blood tests that could facilitate early diagnosis of AAD.

Material and Methods

Subjects

We conducted a retrospective multicentre study to identify the laboratory findings in 137 Norwegian (diagnosed 1978–2016) and 135 Swedish (diagnosed 2000–2013) patients at diagnosis of AAD.

In Norway, informed consent was secured by only including patients registered in The National Addison Registry (ROAS), which covers >75 per cent of all Norwegian patients with AD. In Sweden, patients were included after signing an informed consent to the Swedish Addison Registry [20]. SAR contains clinical data and blood samples from approximately 50% of all Swedish patients with AAD [21]. We ensured population homogeneity by restricting inclusion to confirmed AAD, evidenced by the presence of 21OH-Ab. Adrenalectomy, secondary or transient insufficiency, incomplete medical records or diagnosis before 1978 (Norway) or 2000 (Sweden) led to exclusion.

Information retrieval

Medical records provided patient data and laboratory values at diagnosis. We registered the following categorical variables: sex, acute hospital admission (if yes, administration of intravenous hydrocortisone and fluid), 21OH-Ab, autoimmune polyendocrine syndrome (APS), use of levothyroxine, and in Swedish patients, hyperpigmentation. Clinical and biochemical variables included age, height, weight, blood pressure, S-sodium, S-potassium, B-haemoglobin, S-alanine amino transferase

(ALAT), S-calcium, S-creatinine, S-glucose, random S-cortisol, stimulated S-cortisol, P-adrenocorticotropic hormone (ACTH), S-aldosterone, P-renin activity, P-renin concentration, S-dehydroepiandrosterone sulphate (DHEAS), S-thyroid stimulation hormone (TSH) and thyroid peroxidase autoantibodies (TPO-Ab). Laboratory values obtained after initialization of replacement treatment were excluded, except 21OH-Ab as assays in many cases were performed at a later time-point. Cortisol, ACTH, aldosterone, PRA and renin concentration values marked as 'less than' or 'more than' were entered as the stated number. To investigate the correlation between hypoglycaemia and age, glucose levels were divided into two groups of $\geq$ or <3 mmol L^{-1}. To explore the inhibitory effect of cortisol on TSH levels, 29 of 145 patients were excluded due to ongoing treatment for hypothyroidism. TSH values were then dichotomized as elevated or not.

Laboratory methods have obviously been changed numerous times over the course of 38 years, and methods may have varied between hospitals. We chose to give measured values. When reference ranges clearly differed between hospitals, this is indicated in the text or converted to normal, elevated or low values.

Defining adrenal crisis

There is no universal consensus regarding definition of adrenal crisis, yet systolic blood pressure <100 mmHg is a frequently suggested feature [22–24]. In this study, patients were categorized as having adrenal crisis when meeting the following three criteria: admitted acutely to hospital, found hypotensive (systolic BP < 100 mmHg) and on clinical judgement considered to be in an adrenal crisis.

Statistics

Results are expressed as median [range] or as mean (±standard deviation) when appropriate. The Mann–Whitney independent sample *U*-test was employed to compare differences between groups. Correlation between age and glucose, random cortisol and stimulated cortisol, random cortisol and ACTH, stimulated cortisol and ACTH, and random cortisol and TSH were determined using the Spearman's rank correlation analysis. *P*-values were two-tailed, and significance was considered established at 0.05 for group comparison analyses and correlation of age and glucose, and 0.01 for the remaining correlations.

Results

Subjects

A total number of 272 individuals were included displaying a wide age range (5–79 years) and consisting of more women ($n = 173$) than men ($n = 99$). One-hundred and eighty-seven of 265 (69%) patients were diagnosed during an acute hospital admission, and 78 of 240 (33%) met the criteria of adrenal crisis. Adrenal crisis was associated with debut at slightly younger age (32.6 years (±14.93) vs. 38.4 years (±14.48), $P = 0.04$). One-hundred and thirty-seven of 255 (54%) patients had a concomitant autoimmune disease at diagnosis or have been diagnosed with APS later on. Patient characteristics are summarized in Table 1.

Clinical features at diagnosis

Medical records provided information on body mass index (BMI) in 91 adults. Twenty-two had BMI less than 18.5 kg m^{-2}. There was no significant difference in BMI of patients with (20.1 [13.7–32-3]) compared to those without crisis (20.1 [15.8–34.4], $P = 0.821$). Records of systolic and/or diastolic blood pressures were retrieved from 224 patients. Ninety-three patients (42%) presented with systolic blood pressure <100 mmHg, and 59 (26%) patients had diastolic pressures <60 mmHg. Hyperpigmentation was found in 87% of the Swedish patients at diagnosis.

Table 1 *Patient characteristics*

	Male	Female	Total
No. of patients (%)	99 (36)	173 (64)	272
Median age at diagnosis in years (range)	30 (6–79)	39 (5–77)	36 (5–79)
Diagnosis at acute hospital admission (%)	71 (74)	116 (69)	187 (71)
Adrenal crisis at diagnosis (%)	27 (32)	51 (33)	78 (33)
Patients with APS type I, II (%)	40 (46)	97 (58)	137 (54)

APS, autoimmune polyendocrine syndrome.

Common laboratory findings

Sodium and potassium concentrations were available in 247 and 242 patients, respectively. Sodium below 137 mmol L^{-1} was present in 207 (84%). Potassium was >5.0 mmol L^{-1} in 82 (34%). Eighty-one (34%) exhibited both low sodium and hyperkalaemia (Fig. 1). Only one patient had hyperkalaemia without low sodium. Electrolyte disturbances were significantly more pronounced in patients with an adrenal crisis with median sodium of 127 mmol L^{-1} [101–138] vs. 132 mmol L^{-1} [103–142] ($P < 0.001$), and median potassium of 5.0 mmol L^{-1} [3.5–8.4] vs. 4.5 mmol L^{-1} [3.2–8.6] ($P < 0.001$) compared to those without crisis (Table 2). Eight patients had severe hyperkalaemia (≥7 mmol L^{-1}) and accompanied by severe hyponatraemia (<125 mmol L^{-1}), very low cortisol (<80 nmol L^{-1}) and marked systolic hypotension (<80 mmHg) in all but one. The latter patient had sodium 134 mmol L^{-1}, cortisol 114 mmol L^{-1}, blood pressure 105/60 mmHg, but reached a stimulated cortisol of only 131 nmol L^{-1}.

Hypoglycaemia defined as S-glucose <3 mmol L^{-1} was noted in 15 of 135 patients (type 1 diabetes mellitus (T1D) excluded) of whom two were <18 years of age. In the remaining seven children tested, values ranged from 4.2 to 6.0 mmol L^{-1}. There was a small positive correlation between age and glucose levels, regardless of whether patients with T1D were included (rho = 0.169, $N = 153$, $P = 0.037$) or excluded (rho = 0.172, $N = 135$, $P = 0.047$) (Fig. 2). Both the highest (22.2 mmol L^{-1}) and the lowest (1.1 mmol L^{-1}) values occurred in patients with T1D. The latter was found in a 57-year-old man with ongoing Graves' disease, T1D and asthma. The sudden drop in serum glucose for no apparent reason alerted the physician of possible increased insulin sensitivity due to an underlying AD, and necessary acute treatment and diagnostic workup were performed. Furthermore, a 5-year-old girl presented with serum glucose 1.9 mmol L^{-1}, sodium 127 mmol L^{-1}, potassium 5.4 mmol L^{-1} and calcium 1.93 mmol L^{-1}. She had recently been diagnosed with hypoparathyroidism, and suspicion of AAD was confirmed by cortisol 218 nmol L^{-1} paired with P-ACTH 322 pmol L^{-1}. After initiation of glucocorticoid replacement, calcium fell to 1.48 mmol L^{-1}, which could be due to fluid resuscitation and/or the inhibitory effect of cortisol on intestinal calcium absorption. Noteworthy, two of four siblings have later also been diagnosed with APS type 1.

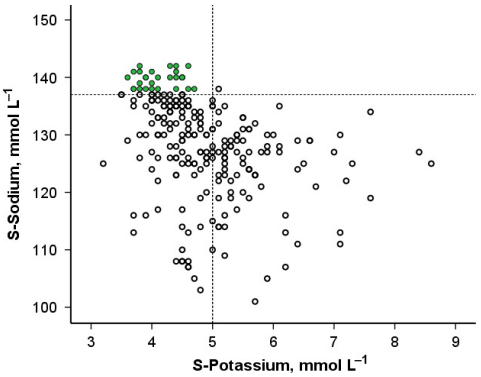


Fig. 1 *Relationship between S-sodium and S-potassium values. Horizontal dotted line depicts lower reference range for sodium, vertical dotted line upper reference range for potassium. Twenty-one patients with both s-sodium > 137 mmol L^{-1} and s-potassium < 5.0 mmol L^{-1} are marked in green (four patients with identical values).*

Mean values of serum haemoglobin, alanine amino transferase (ALAT), calcium, creatinine and serum glucose levels were all well within their respective reference ranges, although aberrant values occurred at both extremes. Median creatinine levels were higher in patients with adrenal crisis compared with those without (91 μmol L^{-1} [46–656] vs. 76 μmol L^{-1} [28–193], $P = 0.003$) (Table 2).

Thyroid function

TSH was measured in 206 patients of which 53 (26%) had documented use of levothyroxine. In patients without known hypothyroidism, TSH was elevated in 79 of 153 (52%). TPO-Ab was only available in 22, but a positive test indicated untreated autoimmune hypothyroidism in eight cases. In the remaining 71, the elevated TSH might have been caused by lack of cortisol as there was a small, but significant negative correlation between TSH and random cortisol (rho = −0.248, $N = 138$, $P = 0.003$) (Fig. 3A).

Assessment of adrenal function

A random serum cortisol value was recorded in 255 patients and was as expected found consistently low (median 62 nmol L^{-1} [1–668]) (Fig. 4A) and

Table 2 *Laboratory values at time of AAD diagnosis*

Laboratory parameter	*N*	Median (range) Adrenal crisis at diagnosis	No adrenal crisis at diagnosis	All	*P*-value*
S-Sodium (mmol L^{-1})	247	127 (101–138)	132 (103–142)	130 (101–142)	<0.001
S-Potassium (mmol L^{-1})	242	5.0 (3.5–8.4)	4.5 (3.2–8.6)	4.6 (3.2–8.6)	<0.001
S-Creatinine (μmol L^{-1})	127	91 (46–656)	76 (28–193)	79 (28–656)	0.003
S-Hb (g dL^{-1})	219	13.8 (7.7–16.8)	13.4 (8.7–17.4)	13.5 (7.7–17.4)	0.770
S-ALAT (U L^{-1})	144	18 (0–144)	16 (0–191)	16 (0–191)	0.369
S-Calcium (mmol L^{-1})	146	2.38 (1.9–3.95)	2.33 (2.0–2.98)	2.33 (1.9–3.95)	0.231
B-Glucose (mmol L^{-1})	153	4.8 (1.1–10.7)	5.1 (1.6–22.2)	5 (1.1–22.2)	0.051
S-TSH (mIE L^{-1})	206	3.4 (0–117)	4.1 (0–180)	4.1 (0–180)	0.709
Random S-cortisol (nmol L^{-1})	255	38 (2–442)	81 (1–668)	62 (1–668)	<0.001
Stimulated S-cortisol (nmol L^{-1})	129	68 (2–437)	117 (11–703)	94 (2–703)	<0.001
P-ACTH (pmol L^{-1})	194	278 (1–1910)	274 (4–1319)	278 (1–1910)	0.054
S-Aldosterone (pmol L^{-1})	97	69 (42–256)	69 (27–230)	69 (27–256)	0.037
PRA (μg L^{-1} h^{-1})	60	9 (3–158)	19 (2–240)	18 (2–240)	0.331
Plasma renin concentration (mIE L^{-1})	24	166 (79–2910)	176 (13–520)	170 (13–2910)	0.868
S-DHEAS (μmol L^{-1})	33	0.4 (0.12–0.9)	0.4 (0.04–2.8)	0.4 (0.04–2.8)	0.453

*Between-group differences as calculated by Mann–Whitney *U*-test
Hb, haemoglobulin; ALAT, alanine amino transferase; TSH, thyroid stimulation hormone; ACTH, adrenocorticotropic hormone; PRA, plasma renin activity; DHEAS, dehydroepiandrosterone sulphate.

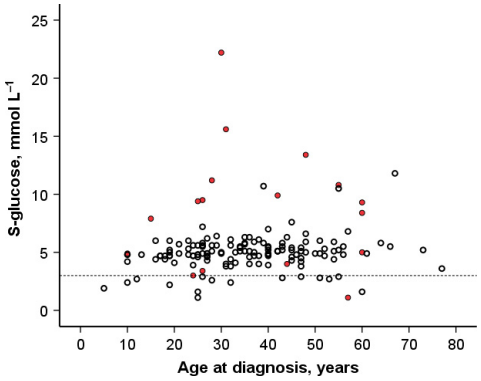


Fig. 2 *Relationship between S-glucose level and age at diagnosis. Dotted horizontal line marks cut-off value for hypoglycaemia, 3.0 mmol L^{-1}. Eighteen patients with type 1 diabetes mellitus are marked in red.*

below 140 nmol L^{-1} in 202 (79%) patients. Conversely, values exceeded 500 nmol L^{-1} in three patients prior to stimulation test. Two used oral contraception (OCP), which is known to increase total cortisol due to higher cortisol-binding globulin (CBG) levels. The third patient suffered from both T1D and primary hypothyroidism and was diagnosed early. Screening revealed ACTH 102 pmol L^{-1} and the presence of 21OH-Ab. Another three patients had values >400 nmol L^{-1}, yet diagnosis was confirmed by minimal response to a cosyntropin stimulation test (cortisol increment ≤58 nmol L^{-1}). All three had clearly elevated ACTH levels and were 21OH-Ab positive.

Cortisol was significantly lower in patients with adrenal crisis (38 nmol L^{-1} [2–442]) compared to those without (81 nmol L^{-1} [1–668], $P < 0.001$) (Table 2). Three of 73 patients in crisis had cortisol >300 nmol L^{-1}, yet all three presented with severe hyponatraemia (110, 121 and 124 mmol L^{-1}). Aldosterone was only measured in the latter two and found low in both (<69 pmol L^{-1} and 75 pmol L^{-1}, respectively).

ACTH was 278 pmol L^{-1} or higher in 99 of 194 patients (50%). Strangely, three patients had values <10 pmol L^{-1} despite positive 21OH-Ab. In one of the patients, subsequent analyses revealed elevated ACTH. Another patient had a cortisol of

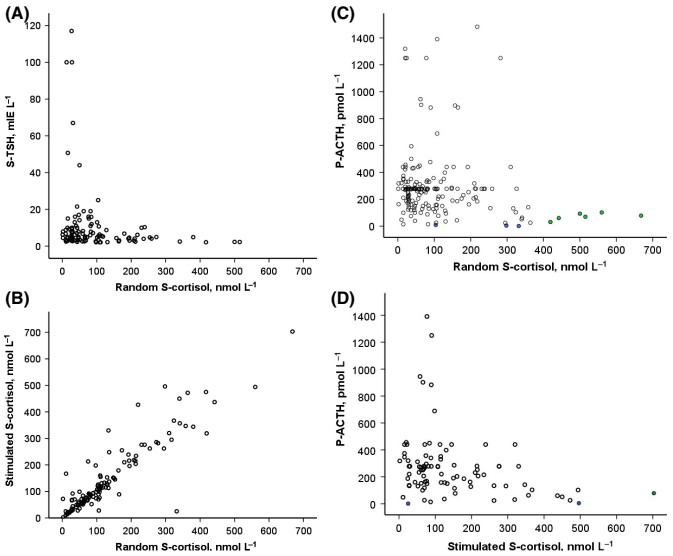


Fig. 3 *(A) Relationship between S-thyroid stimulated hormone (TSH) and random S-cortisol in patients without known concomitant hypothyroidism. (B) Relationship between stimulated S-cortisol and random S-cortisol. (C) Relationship between P-adrenocorticotropic hormone (ACTH) and random S-cortisol. Three patients with ACTH < 10 pmol L^{-1} are marked blue, six patients with cortisol > 400 nmol L^{-1} are marked green. (D) Relationship between P-adrenocorticotropic hormone (ACTH) and stimulated S-cortisol. Two patients with ACTH < 10 pmol L^{-1} are marked blue, one patient with cortisol > 500 nmol L^{-1} is marked green.*

104 nmol L^{-1}, and in yet another, AAD was detected at an early stage due to concomitant hypothyroidism, T1D, vitiligo and pernicious anaemia. There was a small, but significant inverse relationship between random cortisol and ACTH (rho = −0.230, N = 189, P = 0.001) (Fig. 3B). The correlation could be an underestimation because many values were not precisely measured but plotted as 278 pmol L^{-1} when in reality >278 pmol L^{-1}.

A cosyntropin stimulation test was performed in 129 patients (Fig. 4B). Using the cut-off 500 nmol L^{-1}, all but one had a pathological test. The highest stimulated cortisol of 703 nmol L^{-1} belonged to a 27-year-old woman. Of note, the increment in cortisol was minor (from 668) hinting to the presence of excessive CBG levels as noted above. All other clinical and biochemical investigations pointed towards AAD, including elevated ACTH and plasma renin activity, and the presence of 21OH-Ab. It later became apparent that she used OCP. In the remaining 128 patients, cortisol failed to rise above 500 nmol L^{-1} (median peak of 94 nmol L^{-1} [2–703]). In cases where the cosyntropin test was omitted, all patients had cortisol less than 110 nmol L^{-1} and/or ACTH levels elevated above 20 pmol L^{-1}. Overall, there was a strong, positive correlation between stimulated cortisol and random cortisol (rho = 0.884, N = 127, P < 0.001) (Fig. 3B). A moderate, negative correlation was found between ACTH and random cortisol (rho = −0.230, N = 189, P = 0.001) (Fig. 3C) and ACTH and stimulated cortisol (rho = −0.311, N = 92, P = 0.003) (Fig. 3D). All correlations were significant, and the coefficient of determination (rho^2) was 78%, 5% and 10%, respectively.

Aldosterone was measured in 97 patients, and 67 had levels <69 pmol L^{-1}. The highest value noted was 256 pmol L^{-1} and accompanied by cortisol 84 nmol L^{-1}, ACTH 330 pmol L^{-1} and plasma renin activity 65 μg L^{-1} h^{-1}, confirming AD. Median aldosterone value was 69 pmol L^{-1} in both groups. However, more than half (56%) of the

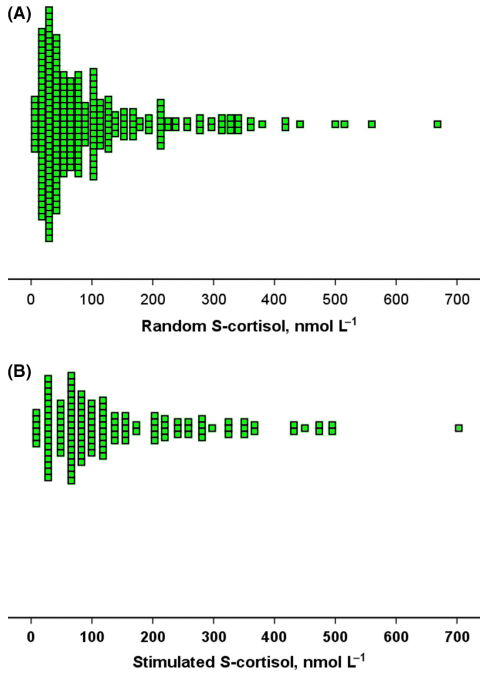


Fig. 4 *(A) Distribution of random S-cortisol values in 255 patients. (B) Distribution of cosyntropin-stimulated S-cortisol in 129 patients.*

measurements were entered as 69, meaning values were not detectable. Aldosterone was <69 in 21 of 26 patients (81%) with crisis, compared to 22 of 61 patients (64%) without. Comparison revealed slightly lower aldosterone in patients with versus without crisis ($P = 0.037$) (Table 2).

Plasma renin activity (PRA) values were elevated in 57 of 60 Norwegian patients, only three exhibited values within the reference range (0.5–3.4 μg L^{-1} h^{-1}). In Sweden, plasma renin concentration was recorded for 24 patients and found elevated >40 mIE L^{-1} in all but one. DHEAS was decreased <2 μmol L^{-1} in 31 of 33 patients.

Discussion

AD often has an insidious presentation with non-specific symptoms that delay diagnosis, often to the point that patients develop an adrenal crisis with risk of fatal outcome. An ongoing challenge is to recognize subtle symptoms and signs of AAD before a life-threatening crisis develop. We show that AAD is associated with low BMI, yet obesity does not rule out the diagnosis. In the Swedish cohort, the vast majority of patients presented with hyperpigmentation, making skin changes an important clinical clue of AD. However, the hyperpigmentation may be subtle, lacking or simply overlooked. This calls for the need for biochemical hints to raise suspicion of AD.

We here show that low sodium is the most consistent routine biochemical finding at diagnosis (Table 3). This adds to the challenge of prompt identification of AAD as sodium disturbances are associated with a plethora of diseases and conditions with multiple aetiologies. Indeed, hyponatraemia is the most common electrolyte abnormality encountered in clinical practice [25]. Low sodium levels are, however, more common in older patients with high morbidity [26, 27]. Although AAD may start at any time of life, the majority of patients are diagnosed between 30 and 50 years of age [2]. We therefore recommend that an otherwise unexplained S-sodium <137 mmol L^{-1} should initiate evaluation for adrenal insufficiency, especially if accompanied by unspecific general symptoms in young- and middle-aged patients.

Of equal importance is the finding that hyperkalaemia only occurs in one-third of patients. Thus, the alleged hallmark of AAD, the combination of low sodium and hyperkalaemia, is only present in a minority. In contrast to common belief, we show that potassium levels hold limited value in AAD workup. Although the presence of hyperkalaemia may substantiate diagnosis, we show that normokalaemia is far more common, thus diminishing the frequently listed significance of potassium aberrations. In short, normokalaemia does not exclude the diagnosis, even in severely ill patients.

Hypoglycaemia occurred in both children and adults, yet the majority of patients were normoglycemic. We found a small, positive correlation between age and glucose levels. Thus, even a low normal glucose could add to suspicion of AAD, especially if seen together with low sodium in younger individuals. In patients with T1D, a sudden drop in insulin requirements or recurrent episodes of hypoglycaemia may be the first biochemical sign of AAD and should prompt further testing of adrenal function [28].

Table 3 *Key biochemical features at diagnosis of autoimmune Addison's disease. The percentages are the fraction of patients fulfilling each criterion.*

Common blood tests
Low sodium (<137 mmol L^{-1}, 84%)
Elevated TSH (52%)
High potassium (>5 mmol L^{-1}, 34%)
Targeted assessment of adrenal function (ref. 9)
Pathological cosyntropin test (S-cortisol <500 nmol L^{-1}, 99%)
Elevated ACTH × 2 upper reference limit (97%)
Elevated plasma renin activity (95%)
Plasma renin concentration (96%)
Low baseline cortisol (<140 nmol L^{-1}, 79%)
Low aldosterone (<67 pmol L^{-1}, 69%)
Other tests
21-hydroxylase autoantibodies (100%)

TSH, thyroid stimulation hormone; ACTH, adrenocorticotropic hormone

Of note, biochemical abnormalities in serum haemoglobin, ALAT and calcium were not consistent indicators of AD. One explanation could be that in general, patients are nowadays diagnosed at an earlier stage than 60 years ago [29], given better availability of hormone assays and the possibility of measuring 21OH-ab, an early biomarker of AAD. Also, interpretation of haemoglobin requires caution, as dehydration may camouflage a low value.

Elevated TSH was frequent, recorded in more than half of the patients who did not use levothyroxine. A high TSH value could indicate untreated hypothyroidism but might also be a sign of unrecognized adrenal insufficiency due to decreased inhibitory effect of cortisol on pituitary TSH production [30]. It is crucial that physicians are aware of the inductive effect of levothyroxine on cortisol metabolism [31]. Indeed, we noticed several cases where initiation of thyroxine therapy led to worsening of the clinical condition [32], even precipitating an adrenal crisis. Irrespective of cause, elevated TSH accompanied by low sodium should trigger consideration of AD.

We show that aberrancy in cortisol, ACTH, aldosterone, plasma renin activity and concentration, and DHEAS values are reliable markers of AD. Once suspected, diagnosis of AAD is often easily confirmed by targeted investigations. Notably, we show that a low random cortisol value is strongly and significantly associated with a low stimulated cortisol value. However, normal cortisol does not rule out AD. Also, we here demonstrate the potential deceptive effect of OCP in elevating CBG-bound cortisol. The lack of increment in cortisol by cosyntropin can give a hint of an underlying undiagnosed AD and underlines the importance of obtaining a careful medication history. Equally important, practically all patients presented with elevated ACTH. The vast majority also exhibited aberrant values of aldosterone and renin. We therefore recommend a low threshold for measurement of ACTH, aldosterone and renin in addition to cortisol upon suspicion of AAD.

There is no universal consensus regarding definition of adrenal crisis, although a number of proposals have been put forward. The most recent definition [24] requires 'an acute deterioration in health' and hypotension relieved following parenteral glucocorticoid administration. Here, we defined adrenal crisis as patients admitted acutely to hospital, found hypotensive (systolic BP <100 mmHg), and on clinical judgement considered to be in an adrenal crisis. In our cohort, more than 70% were diagnosed in relation to acute hospital admission. Less than half of these presented with hypotension. Thus, our finding of 33% in crisis at debut may be an underestimate. Importantly, one patient presented with cortisol <1 nmol L^{-1} and sodium 103 mmol L^{-1}, and another patient had a cortisol of 50 nmol L^{-1} and potassium of 8.6 mmol L^{-1} at diagnosis. Both patients were acutely admitted to hospital but failed to be defined as in an adrenal crisis as the blood pressure was >100 mmHg. We therefore recommend that systolic blood pressure <100 mmHg be considered indicative of but not mandatory for adrenal crisis.

Additional data from the registries revealed that more than half of patients had at least one other autoimmune endocrine disorder, either present at debut of AAD or acquired later in life. Thus, physicians should be aware of the increased risk of AAD in conjunction with other organ-specific autoimmune disorders, and we advocate a low threshold for testing adrenal function in these patients.

Assay of 21OH-Ab is probably the earliest indication of a developing AAD. In patients with autoimmune disease and unexplained vague symptoms

such as fatigue or abdominal symptoms, screening for 21OH-Ab is warranted and may secure an early diagnosis of AAD. Moreover, early detection of 21OH-Ab is useful to identify possible candidates for immunosuppressive therapy aimed at reversing and even curing AAD [33].

Diagnostic accuracy of AAD was ensured by scrutiny of patient records and solely including patients who had received follow-up treatment and care of his or her AAD over time. Unfortunately, the true prevalence of AAD is not known for all of the participating centres, and we were therefore unable to estimate the proportion of patients included. Still, the ROAS registry in participating centers covers the vast majority of Norwegian patients with AAD (>75%), and in Sweden, virtually all invited patients agreed to participate. Also, the relatively high number of included subjects, recruitment from multiple hospitals and different decades, all contribute to reduce selection bias to a minimum and increase the generalizability of our findings.

However, the retrospective, multi-centred design is susceptible to various biases. First, it offers limited control with reported data. In most cases, medical records did not provide information on the time of blood sampling, thus possibly confounding the interpretation. This was especially true for cortisol and ACTH measurements. Although cortisol and ATCH reveal clear circadian variation, it can be assumed that this is largely lost in AAD when the adrenal cortex is continuously stressed by high ACTH. Secondly, the laboratory methods and assays have changed in the course of 38 years and there are variations between the laboratories. Thirdly, not all parameters of interest were recorded for every patient. However, even with these weaknesses, our results from a large collection of patients reflect real-world data, and the information clinicians will encounter when evaluating their patients.

In conclusion, low sodium was the only consistent finding amongst routine blood tests, independent of degree of adrenal insufficiency. Elevated TSH was present in more than half of the patients. High potassium, however, only occurred in one-third. We urge that low sodium and or elevated TSH without obvious explanation should trigger consideration of AAD, and on clinical suspicion bring about assay of a paired cortisol and ACTH. Importantly, initiation of levothyroxine can precipitate deterioration in the clinical condition and even induce an adrenal crisis. Early detection of AAD is vital, as delay in diagnosis put patients at risk of lethal adrenal crisis.

Acknowledgements

The study was supported by the Regional Health Authorities of Western Norway, University of Bergen, The Norwegian Research council, The Swedish Research Council, Torsten and Ragnar Söderberg Foundations, The regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet, the Swedish Society for Medical Research, the Swedish Society of Medicine, Tore Nilson's Foundation for Medical Research, and the Åke Wiberg Foundation.

Conflict of interest statement

The author reports no conflicts of interest in this work.

The manuscript has been handled by an external editor, Professor Olov Wiklund, Department of Molecular and Clinical Medicine at Institute of Medicine, Sahlgrenska University Hospital Gothenburg, Sweden.

References

1 Bensing S, Hulting AL, Husebye ES, Kampe O, Lovas K. MANAGEMENT OF ENDOCRINE DISEASE: Epidemiology, quality of life and complications of primary adrenal insufficiency: a review. *Eur J Endocrinol* 2016; **175:** R107–16.
2 Charmandari E, Nicolaides NC, Chrousos GP. Adrenal insufficiency. *Lancet* 2014; **383:** 2152–67.
3 Eriksson D, Bianchi M, Landegren N *et al.* Extended exome sequencing identifies BACH2 as a novel major risk locus for Addison's disease. *J Intern Med* 2016; **280:** 595–608.
4 Puar TH, Stikkelbroeck NM, Smans LC, Zelissen PM, Hermus AR. Adrenal crisis: still a deadly event in the 21st century. *Am J Med* 2016; **129:** 339.e1–339.e9.
5 Mason AS, Meade TW, Lee JA, Morris JN. Epidemiological and clinical picture of Addison's disease. *Lancet* 1968; **2:** 744–7.
6 Papierska L, Rabijewski M. Delay in diagnosis of adrenal insufficiency is a frequent cause of adrenal crisis. *Int J Endocrinol* 2013; **2013:** 5 482370.
7 Bleicken B, Hahner S, Ventz M, Quinkler M. Delayed diagnosis of adrenal insufficiency is common: a cross-sectional study in 216 patients. *Am J Med Sci* 2010; **339:** 525–31.
8 Bjornsdottir S, Sundstrom A, Ludvigsson JF, Blomqvist P, Kampe O, Bensing S. Drug prescription patterns in patients with Addison's disease: a Swedish population-based cohort study. *J Clin Endocrinol Metab* 2013; **98:** 2009–18.
9 Bornstein SR, Allolio B, Arlt W *et al.* Diagnosis and treatment of primary adrenal insufficiency: an endocrine society clinical

practice guideline. *J Clin Endocrinol Metab* 2016; **101:** 364–89.
10 Eisenbarth GS, Gottlieb PA. Autoimmune polyendocrine syndromes. *N Engl J Med* 2004; **350:** 2068–79.
11 Husebye ES, Allolio B, Arlt W *et al.* Consensus statement on the diagnosis, treatment and follow-up of patients with primary adrenal insufficiency. *J Intern Med* 2014; **275:** 104–15.
12 Wass JA, Arlt W. How to avoid precipitating an acute adrenal crisis. *BMJ* 2012; **345:** e6333.
13 Arlt W, Callies F, van Vlijmen JC *et al.* Dehydroepiandrosterone replacement in women with adrenal insufficiency. *N Engl J Med* 1999; **341:** 1013–20.
14 Salpietro V, Polizzi A, Di Rosa G *et al.* Adrenal disorders and the paediatric brain: pathophysiological considerations and clinical implications. *Int J Endocrinol* 2014; **2014:** 282489.
15 Samaan NA. Hypoglycemia secondary to endocrine deficiencies. *Endocrinol Metab Clin North Am* 1989; **18:** 145–54.
16 Hsieh S, White PC. Presentation of primary adrenal insufficiency in childhood. *J Clin Endocrinol Metab* 2011; **96:** E925–8.
17 Coco G, Dal Pra C, Presotto F *et al.* Estimated risk for developing autoimmune Addison's disease in patients with adrenal cortex autoantibodies. *J Clin Endocrinol Metab* 2006; **91:** 1637–45.
18 Betterle C, Coco G, Zanchetta R. Adrenal cortex autoantibodies in subjects with normal adrenal function. *Best Pract Res Clin Endocrinol Metab* 2005; **19:** 85–99.
19 Erichsen MM, Lovas K, Skinningsrud B *et al.* Clinical, immunological, and genetic features of autoimmune primary adrenal insufficiency: observations from a Norwegian registry. *J Clin Endocrinol Metab* 2009; **94:** 4882–90.
20 Galbois A, Rudler M, Massard J *et al.* Assessment of adrenal function in cirrhotic patients: salivary cortisol should be preferred. *J Hepatol* 2010; **52:** 839–45.
21 Dalin F, Nordling Eriksson G, Dahlqvist P *et al.* Clinical and immunological characteristics of Autoimmune Addison's disease: a nationwide Swedish multicenter study. *J Clin Endocrinol Metab* 2016; **102:** 379–89.
22 Hahner S, Spinnler C, Fassnacht M *et al.* High incidence of adrenal crisis in educated patients with chronic adrenal insufficiency: a prospective study. *J Clin Endocrinol Metab* 2015; **100:** 407–16.
23 Allolio B. Extensive expertise in endocrinology. Adrenal crisis. *Eur J Endocrinol* 2015; **172:** R115–24.
24 Rushworth RL, Torpy DJ, Falhammar H. Adrenal crises: perspectives and research directions. *Endocrine* 2017; **55:** 336–45.
25 Verbalis JG, Goldsmith SR, Greenberg A *et al.* Diagnosis, evaluation, and treatment of hyponatremia: expert panel recommendations. *Am J Med* 2013; **126(10 Suppl 1):** S1–42.
26 Hawkins RC. Age and gender as risk factors for hyponatremia and hypernatremia. *Clin Chim Acta* 2003; **337:** 169–72.
27 Mohan S, Gu S, Parikh A, Radhakrishnan J. Prevalence of hyponatremia and association with mortality: results from NHANES. *Am J Med* 2013; **126:** 1127–1137.e1121.
28 Likhari T, Magzoub S, Griffiths MJ, Buch HN. Screening for Addison's disease in patients with type 1 diabetes mellitus and recurrent hypoglycaemia. *Postgrad Med J* 2007; **83:** 420–1.
29 Berlin R. Addison's disease: familial incidence and occurrence in association with pernicious anemia. *Acta Med Scand* 1952; **144:** 1–6.
30 Hangaard J, Andersen M, Grodum E, Koldkjaer O, Hagen C. Pulsatile thyrotropin secretion in patients with Addison's disease during variable glucocorticoid therapy. *J Clin Endocrinol Metab* 1996; **81:** 2502–7.
31 Hahner S, Loeffler M, Bleicken B *et al.* Epidemiology of adrenal crisis in chronic adrenal insufficiency: the need for new prevention strategies. *Eur J Endocrinol* 2010; **162:** 597–602.
32 Murray JS, Jayarajasingh R, Perros P. Deterioration of symptoms after start of thyroid hormone replacement. *Br Med J* 2001; **323:** 332–3.
33 Pearce SH, Mitchell AL, Bennett S *et al.* Adrenal steroidogenesis after B lymphocyte depletion therapy in new-onset Addison's disease. *J Clin Endocrinol Metab* 2012; **97:** E1927–32.

Correspondence: Åse Bjorvatn Sævik, Department of Clinical Science, University of Bergen, Haukeland University Hospital, N-5021 Bergen, Norway.
(fax: +4755972950; e-mail: aasebjorvatn@hotmail.com)

Residual Corticosteroid Production in Autoimmune Addison Disease

Åse Bjorvatn Sævik,[1,2] Anna-Karin Åkerman,[3,4] Paal Methlie,[1,2,5] Marcus Quinkler,[6] Anders Palmstrøm Jørgensen,[7] Charlotte Höybye,[4,8] Aleksandra J. Debowska,[9] Bjørn Gunnar Nedrebø,[1,10] Anne Lise Dahle,[10] Siri Carlsen,[11] Aneta Tomkowicz,[12] Stina Therese Sollid,[13] Ingrid Nermoen,[14] Kaja Grønning,[14] Per Dahlqvist,[15] Guri Grimnes,[16,17] Jakob Skov,[4] Trine Finnes,[18] Susanna F Valland,[18] Jeanette Wahlberg,[19] Synnøve Emblem Holte,[20] Katerina Simunkova,[1] Olle Kämpe,[2,8,21] Eystein Sverre Husebye,[1,2,5,21] Sophie Bensing,[4,8] and Marianne Øksnes,[1,2,5,21]

[1]Department of Clinical Science, University of Bergen, Norway; [2]K.G. Jebsen Center for Autoimmune Disorders, University of Bergen, Bergen, Norway; [3]Department of Medicine, Örebro University Hospital, Örebro, Sweden; [4]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; [5]Department of Medicine, Haukeland University Hospital, Bergen, Norway; [6]Endocrinology in Charlottenburg, Berlin, Germany; [7]Department of Endocrinology, Oslo University Hospital, Oslo, Norway; [8]Department of Endocrinology, Metabolism and Diabetes, Karolinska University Hospital, Stockholm, Sweden; [9]Department of Medicine, Vestfold Hospital Trust, Tønsberg, Norway; [10]Department of Internal Medicine, Haugesund Hospital, Haugesund, Norway; [11]Department of Endocrinology, Stavanger University Hospital, Stavanger, Norway; [12]Department of Medicine, Sørlandet Hospital, Kristiansand, Norway; [13]Department of Medicine, Drammen Hospital, Vestre Viken Health Trust, Drammen, Norway; [14]Department of Endocrinology, Akershus University Hospital, Lørenskog, Norway; [15]Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden; [16]Division of Internal Medicine, University Hospital of North Norway, Tromsø, Norway; [17]Tromsø Endocrine Research Group, Department of Clinical Medicine, UiT the Arctic University of Norway, Tromsø, Norway; [18]Section of Endocrinology, Innlandet Hospital Trust, Hamar, Norway; [19]Department of Endocrinology and Department of Health, Medicine and Caring Sciences, Linköping University, Linköping, Sweden; [20]Department of Medicine, Sørlandet Hospital, Arendal, Norway; and [21]Department of Medicine (Solna), Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden

ORCiD numbers: 0000-0002-5981-6800 (Å. B. Sævik); 0000-0003-4028-1671 (M. Quinkler); 0000-0002-1246-9194 (A. P. Jørgensen); 0000-0002-3980-1927 (C. Höybye); 0000-0003-4016-7502 (A. J. Debowska); 0000-0003-1635-8325 (B. G. Nedrebo); 0000-0001-8153-938X (S. T. Sollid); 0000-0002-6471-9503 (P. Dahlqvist); 0000-0003-2292-9489 (G. Grimnes); 0000-0002-3738-1367 (J. Skov); 0000-0003-1102-8706 (T. Finnes); 0000-0003-4061-6830 (J. Wahlberg); 0000-0001-6091-9914 (O. Kämpe); 0000-0002-7886-2976 (E. S. Husebye); 0000-0002-9193-2860 (S. Bensing).

Context: Contrary to current dogma, growing evidence suggests that some patients with autoimmune Addison disease (AAD) produce corticosteroids even years after diagnosis.

Objective: To determine frequencies and clinical features of residual corticosteroid production in patients with AAD.

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received 31 January 2020. Accepted 7 May 2020.
First Published Online 11 May 2020.
Corrected and Typeset 5 June 2020.

Abbreviations: AAD, autoimmune Addison disease; ACTH, adrenocorticotropic hormone; AddiQoL, AD-specific QoL questionnaire; APS2, autoimmune polyendocrine syndrome type 2; BMI, body mass index; CI, confidence interval; FC, fludrocortisone; GC, glucocorticoid; HRQoL, health-related quality of life; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MC, mineralocorticoid; OR, odds ratio; PRC, plasma renin concentration; RAND, HRQoL survey; SD, standard deviation; TART, testicular adrenal rest tumor.

Design: Two-staged, cross-sectional clinical study in 17 centers (Norway, Sweden, and Germany). Residual glucocorticoid (GC) production was defined as quantifiable serum cortisol and 11-deoxycortisol and residual mineralocorticoid (MC) production as quantifiable serum aldosterone and corticosterone after > 18 hours of medication fasting. Corticosteroids were analyzed by liquid chromatography–tandem mass spectrometry. Clinical variables included frequency of adrenal crises and quality of life. Peak cortisol response was evaluated by a standard 250 µg cosyntropin test.

Results: Fifty-eight (30.2%) of 192 patients had residual GC production, more common in men (n = 33; $P < 0.002$) and in shorter disease duration (median 6 [0-44] vs 13 [0-53] years; $P < 0.001$). Residual MC production was found in 26 (13.5%) patients and associated with shorter disease duration (median 5.5 [0.5-26.0] vs 13 [0-53] years; $P < 0.004$), lower fludrocortisone replacement dosage (median 0.075 [0.050-0.120] vs 0.100 [0.028-0.300] mg; $P < 0.005$), and higher plasma renin concentration (median 179 [22-915] vs 47.5 [0.6-658.0] mU/L; $P < 0.001$). There was no significant association between residual production and frequency of adrenal crises or quality of life. None had a normal cosyntropin response, but peak cortisol strongly correlated with unstimulated cortisol (r = 0.989; $P < 0.001$) and plasma adrenocorticotropic hormone (ACTH; r = –0.487; $P < 0.001$).

Conclusion: In established AAD, one-third of the patients still produce GCs even decades after diagnosis. Residual production is more common in men and in patients with shorter disease duration but is not associated with adrenal crises or quality of life. (***J Clin Endocrinol Metab*** **105: 2430–2441, 2020)**

Key Words: Adrenal failure; adrenal steroids; Autoimmune Addison disease; cortisol; primary adrenal insufficiency; residual function

Autoimmune Addison disease (AAD) is generally considered to be irreversible, inevitably leading to total destruction of the functional adrenal cortex (1). However, increasing evidence indicates that a subgroup of patients retain some level of corticosteroid production even after many years of disease duration.

In 2011, Smans and Zelissen found quantifiable baseline cortisol levels in 7 of 27 patients with established AAD, measured in a medication fasting state (2). More recently, Vulto et al reported measurable levels of the cortisol precursor, 11-deoxycortisol, in 8 of 20 patients with primary adrenal insufficiency (3). Efforts to exploit residual production therapeutically have demonstrated partial improvement in peak cortisol response to cosyntropin stimulation testing in 7 of 13 patients with newly diagnosed AAD after 12 weeks combined treatment with rituximab and depot tetracosactide (4). In 4 of these patients, stimulated serum cortisol exceeded 100 nmol/L after 72 weeks. At study start, these 4 patients had higher mean stimulated cortisol levels, but did otherwise not differ from the 9 other patients.

Up until now, studies have been performed only in small cohorts, and the clinical relevance of residual production has not yet been addressed. Residual glucocorticoid (GC) production could partly explain observed discrepancies in outcome for patients with AAD. Clinical experience shows great differences in dosage needs for GC replacement therapy, and not all patients require mineralocorticoid (MC) replacement (5). Moreover, 50% of patients with AAD have never experienced an adrenal crisis, and 10% have never required extra GC doses (6). Finally, there are large variations in self-assessed health-related quality of life (HRQoL) in AAD that could potentially be attributed to residual production (7, 8).

Here, we aimed to determine the frequency of residual corticosteroid production in established AAD and to examine the clinical features of residual production.

Material and Methods

Participants

We recruited study participants among patients enrolled in the Norwegian Registry of Organ-Specific Autoimmune Diseases, the Swedish Addison Registry, and patients receiving follow-up at the endocrine center "Endokrinologie in Charlottenburg" in Berlin, Germany. Invitation letters were sent to eligible candidates by mail or handed out at regular clinical visits. All included participants had confirmed autoimmune etiology with presence of 21-hydroxylase antibodies, were prescribed GC replacement therapy, and were between 18 and 75 years of age at screening. Exclusion criteria were diabetes mellitus type 1, cancer, severe organ failure, pregnancy, lactation, and current use of medications with known pharmaceutical interactions with adrenocortical hormones (antiepileptics, rifampicin, St John's wart). Any comorbidity had to be stable for at least 3 months before inclusion.

Only patients on hydrocortisone or cortisone acetate replacement therapy were included. Patients previously using dual-release hydrocortisone were switched to cortisone acetate or hydrocortisone at least 1 week prior to blood sampling. Any dehydroepiandrosterone treatment was paused for at least 1 week; alternatively androgen measurements were excluded from statistical analyses. Use of prednisolone or exogenous GCs on indication(s) other than adrenal insufficiency was paused for at least 3 months before blood sampling. Patients using any other antihypertensive medication(s) than alpha blockers or calcium channel blockers, including diuretics, were excluded from analyses on electrolytes, renin, and MC hormones. Patients were instructed to abstain from grapefruit juice and licorice for at least 1 week and caffeinated drinks for at least 24 hours before blood sampling.

Study design

From September 2018 through January 2020 we performed a 2-staged, cross-sectional multicenter clinical study comprising patients with AAD at 17 hospitals in Norway, Sweden, and Germany (Fig. 1). All authors vouch for the accuracy of the data and for the fidelity of the study protocol.

Written informed consent was obtained from all participants before study entry. At stage 1, we registered patient characteristics including age, sex, disease duration, medications, self-reported frequency of adrenal crises and infections, comorbidities, autoimmune polyendocrine syndrome type 2 (APS2), disease-related symptoms, physical health (body mass index [(BMI], blood pressure, and presence of hyperpigmentation), and HRQoL questionnaires. All participants were prescribed hydrocortisone for intramuscular use and instructed to take their replacement medications upon symptoms of precipitating adrenal crisis. Thereafter, patients returned on an agreed morning for medication fasting blood sampling after abstaining from GC and MC intake not later than 2 PM and 8 AM the day before, respectively.

At stage 2, patients with quantifiable levels of serum cortisol and 11-deoxycortisol and/or quantifiable levels of serum aldosterone and corticosterone were asked to return for a standard 250 µg cosyntropin stimulation test (Synacthen). Blood samples were collected before (0 minutes) and 30 and 60 minutes after intravenous injection of cosyntropin. Participants with a long commute to the hospital were offered to combine screening and stimulation testing on the same day. At Haukeland University Hospital, we also invited all patients without quantifiable serum cortisol and 11-deoxycortisol to serve as negative controls. Before testing, patients abstained from their steroid replacement therapy in the same manner as described above. A normal response was defined as peak cortisol exceeding 412 or 485 nmol/L after 30 or 60 minutes, respectively (9). The peak response was defined as the highest serum cortisol value recorded at either 30 or 60 minutes.

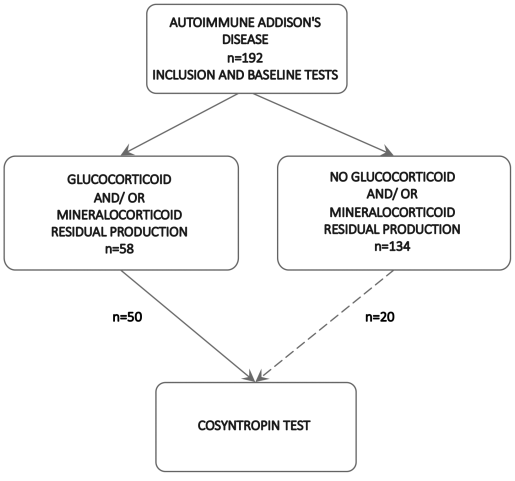


Figure 1. Flow chart of study procedures.

Outcomes

The primary endpoint was frequency of residual GC and/or MC production in patients with AAD. Secondary endpoints included comparison of patients with and without residual GC and/or MC production with regards to patient characteristics including age, sex, disease duration, steroid replacement therapy, peak cortisol in cosyntropin testing, frequency of adrenal crises and infections, physical health (BMI, blood pressure, presence of hyperpigmentation), and HRQoL.

Laboratory tests

Routine blood tests were analyzed locally: hemoglobin, glycated hemoglobin, thyroid-stimulating hormone, free thyroxine, cobalamin, ferritin, creatinine, sodium, potassium, cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, thyroid peroxidase antibodies, ACTH, and plasma renin concentration (PRC). Levels of ACTH exceeding the upper limit of quantification were plotted as 278 pmol/L. All corticosteroid analyses were performed at Haukeland University Hospital by a liquid chromatography–tandem mass spectroscopy (LC-MS/MS) assay further developed from and expanded on a published method (10), measuring cortisol, 11-deoxycortisol, 21-deoxycortisol, cortisone, 18-oxocortisol, 18-hydroxycortisol, tetrahydrocortisol, allo-tetrahydrocortisol, tetrahydrocortisone, allo-tetrahydrocortisone, aldosterone, corticosterone, 11-deoxycorticosterone, androstendione, testosterone, epitestosterone, dihydrotestosterone, and progesterone (Fig. 2). The lower limit of quantification for each corticosteroid is listed in Table 1.

Defining residual corticosteroid production

There is no consensus on the definition of residual corticosteroid production, and no marker of endogenous GC or MC production exists. Here, we defined residual GC production as quantifiable levels of serum cortisol (>0.914 nmol/L) and 11-deoxycortisol (>0.114 nmol/L) and residual MC production as quantifiable levels of serum aldosterone (> 8 pmol/L) and corticosterone (>0.114 nmol/L). All blood samples were obtained in the morning after at least 18 hours without hydrocortisone or cortisone acetate and at least 24 hours without fludrocortisone (FC).

HRQoL questionnaires

All patients filled out 1 generic (RAND-36) (11) and 1 AAD-specific (AddiQoL) (12) questionnaire assessing HRQoL. RAND-36 is a license free version of the Short Form 36-item (SF-36). It comprises 36 items assessing 8 health concepts:

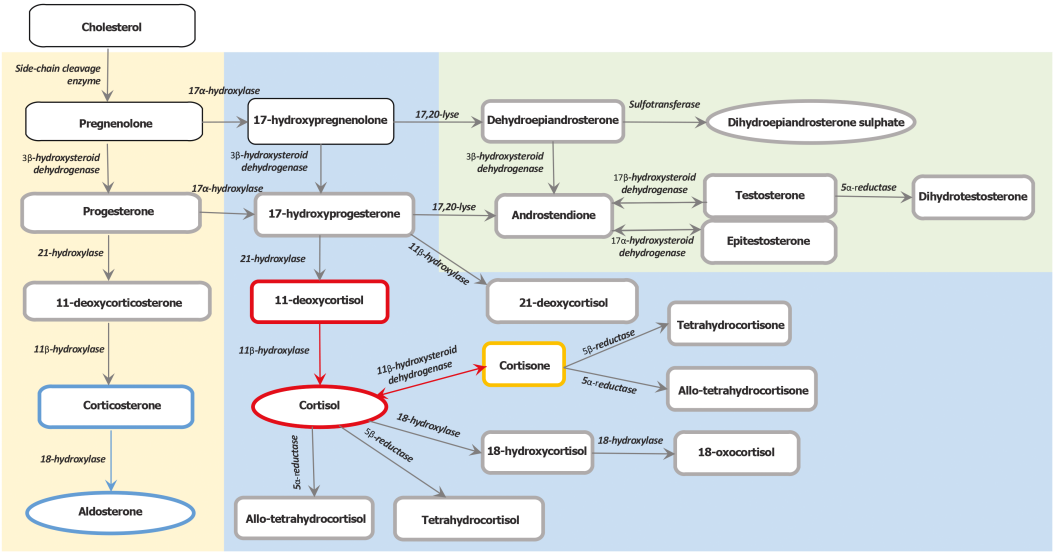


Figure 2. Synthesis of adrenocortical steroids. The 3 main adrenocortical steroids (aldosterone, cortisol, and dihydroepiandrostendione sulphate) are shown in circles, while precursor steroids and metabolites are shown in rectangles. Bold borders mark steroids analyzed in this study. Cortisol and 11-deoxycortisol define residual glucocorticoid production and are marked in red. Aldosterone and corticosterone define residual mineralocorticoid production and are marked in blue. Red and blue arrows mark the enzymatic reactions for activation of cortisol and aldosterone, respectively. Cortisone is both a metabolite and precursor of cortisol and is marked in yellow.

physical functioning, role limitations caused by physical health problems, role limitations caused by emotional problems, social functioning, general mental health, vitality, bodily pain, and general health. Scoring of RAND-36 is a 2-step process. First, precoded numeric values are recorded to a number between 0 and 100 where a higher score represents a better health state. In the second step, items belonging to the same health concept are averaged to create 1 of the 8 total scores (11). AddiQoL has been validated and translated into several languages including Norwegian, Swedish, and German (12). The questionnaire contains 30 items divided into 4 domains: fatigue, emotional well-being, adrenal insufficiency-related symptoms, and miscellaneous (sexuality, sleep, and impact of intercurrent disease). Every item has 6 scoring categories scored as 1, 2, 2, 3, 3, and 4 for positive statements and 4, 3, 3, 2, 2, and 1 for negative statements. A total score is generated by adding the score of individual items, producing a total score ranging from 30 to 120 where a higher score indicates a more favorable HRQoL. A missing individual item score can be replaced by the mean score from the rest of the items in the same subdimension.

Statistics

We report the primary endpoint as absolute numbers and percentages. Descriptive statistics and secondary endpoints are presented as numbers and percentages for categorical data and as medians and range [minimum to maximum] or as means and standard deviations (± SD) for continuous variables. To compare subgroups, we used independent samples *t* test, Mann-Whitney independent sample U test, and chi-square test, as appropriate. Correlations were explored using the Spearman rank correlation. Binary logistic regression was performed to assess the impact of key patient characteristics on the likelihood of having residual GC or MC production. Nine clinically relevant variables were included: age at diagnosis, sex, disease duration, history of adrenal crisis ever, BMI, hydrocortisone-equivalent dosage (mg cortisone acetate/1.25 = mg hydrocortisone), FC dosage, AddiQoL-30 score, and plasma ACTH (for GC) or PRC (for MC). Preliminary analyses were conducted to ensure no violation of the assumption of multicollinearity. Results are presented as odds ratio (OR) and 95% confidence interval (CI). To reduce the risk of type I error, the alpha value was set to 0.01.

Ethics

Ethical approval was granted from all participating countries before study start, by the Regional Ethical Committee of South-East Norway (permit no. 2018/751/REK Sør-Øst), of Stockholm, Sweden (permit no. 2018/2247-32), and of Berlin, Germany (permit no. Eth-47/18). The study was registered at clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT03793114) and conducted in agreement with local and international guidelines and regulations, including the Declaration of Helsinki (2013 version) and the principles of good clinical practice (CPMP/ICH/135/95).

Results

Stage 1: Frequency and clinical characteristics of residual corticosteroid production

Frequency of residual production. We included 197 patients with AAD. Five patients declined to proceed to medication fasting blood sampling and were excluded from the study. Baseline characteristics for the remaining

Table 1. Corticosteroids in Patients with and Residual Glucocorticoid Production

Corticosteroid	N	LLoQ	Median (minimum-maximum) GC+	Median (minimum-maximum) GC–	*P*
18-oxo-cortisol (nmol/L)	192	0.046	0.00 (0.00-0.30)	0.00 (0.00-1.27)	<0.001[a]
18-OH-cortisol (nmol/L)	192	0.046	0.26 (0.00-0.28)	0.00 (0.00-0.20)	<0.001[a]
Aldosterone (pmol/L)[b]	191	8.0	0 (0-220)	0 (0-25)	<0.001[a]
Cortisone (nmol/L)	191	0.914	10.21 (1.63-46.88)	0.00 (0.00-4.16)	<0.001[a]
Cortisol (nmol/L)[c]	192	0.914	57.28 (5.48-507.04)	0.98 (0.00-27.18)	<0.001[a]
DHEAS (nmol/L)	176	22.9	432.69 (25.07-2400.12)	0.00 (0.00-1459.51)	<0.001[a]
21-deoxycortisol (nmol/L)	192	0.023	0.032 (0.00-14.50)	0.00 (0.00-1.05)	<0.001[a]
Corticosterone (nmol/L)	191	0.114	3.50 (0.00-50.84)	0.00 (0.00-2.67)	<0.001[a]
Allo-tetrahydrocortisol (nmol/L)	191	0.114	2.14 (0.00-21.54)	0.00 (0.00-1.56)	<0.001[a]
11-deoxycortisol (nmol/L)	192	0.114	0.60 (0.12-2.87)	0.00 (0.00-0.21)	<0.001[a]
Tetrahydrocortisol (nmol/L)	192	0.343	1.57 (0.00-17.06)	0.00 (0.00-2.84)	<0.001[a]
Allo-tetrahydrocortisone (nmol/L)	192	0.343	0.00 (0.00-1.39)	0.00 (0.00–0.42)	<0.001[a]
Tetrahydrocortisone (nmol/L)	192	0.114	0.95 (0.00–9.82)	0.00 (0.00-0.69)	<0.001[a]
Androstendione (nmol/L)	175	0.023	0.92 (0.00-4.51)	0.440 (0.00-4.04)	<0.001[a]
11-deoxycorticosterone (nmol/L)	191	0.023	0.12 (0.00-0.94)	0.00 (0.00-0.16)	<0.001[a]
Testosterone (nmol/L)	176	0.023	7.74 (0.04-27.39)	0.34 (0.00-30.57)	<0.001[a]
DHEA (nmol/L)	174	0.617	0.71 (0.00-4.33)	0.34 (0.00-1.97)	<0.001[a]
17-hydroxy-progesterone (nmol/L)	192	0.114	2.90 (0.00-49.29)	0.73 (0.00-894.6)	<0.001[a]
Epitestosterone (nmol/L)	176	0.023	0.06 (0.00-0.31)	0.00 (0.00-0.46)	0.008[a]
Dihydrotestosterone (nmol/L)	176	0.206	0.57 (0.00-2.50)	0.00 (0.00-2.61)	0.020
Progesterone (nmol/L)	191	0.114	0.18 (0.00-81.35)	0.00 (0.00-48.27)	<0.001[a]

GC+, residual glucocorticoid production; GC–, no residual glucocorticoid production;
Abbreviations: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; GC, glucocorticoid; LLoQ, lower limit of quantification.
[a]Statistically significant at 0.01 level.
[b]To convert serum aldosterone values (pmol/L) to ng/dL, divide by 27.7.
[c]To convert serum cortisol values (nmol/L) to µg/dL, divide by 27.6.

192 patients are presented in Table 2. The medication fast was generally well-tolerated, with only a few individuals reporting increased tiredness and/or headache at blood sampling. Fifty-eight (30.2%) patients had quantifiable levels of serum cortisol and 11-deoxycortisol (Fig. 3A, B), and 26 (13.5%) patients had quantifiable levels of serum aldosterone and corticosterone (Fig. 3C, D). In 24 (12.5%) patients, all 4 hormones were quantifiable. There was a strong positive correlation between serum cortisol and 11-deoxycortisol levels (r = 0.796; P < 0.001) (Fig. 4A), as well as for aldosterone and corticosterone (r = 0.605; P < 0.001) (Fig. 4B).

Residual GC production. Thirty-three (56.9%) of the 58 patients with residual GC production were men ($X^2(1, N = 192) = 9.405$; $P < 0.002$). Patients with residual GC production also had significantly shorter disease duration (median 6 [0-44] vs 13 [0-53] years; $P < 0.001$) and higher levels of all adrenal steroids except 18-oxo-cortisol (Table 1). These findings were supported by binary logistic regression, where male sex (OR 5.9; 95% CI, 2.4-14.5; $P < 0.001$) and short disease duration both predicted residual GC production (OR 0.95; 95% CI, 0.91-0.98; $P < 0.006$). As a whole, the regression model explained between 18.5% and 26.3% of the variance in residual GC production status and correctly classified 75.3% of the cases ($X^2(9, N = 182) = 37.308$; $P < 0.001$).

Table 2. Patient characteristics (n = 192)

Characteristics	Number (%) or Median (range) or Mean (±SD)
Female	116 (60.4)
Age (years)	48.3 ± 13.0
Age at diagnosis, years	33.5 (11-64)
Disease duration, years	11 (0-53)
APS 2, n (%)	109 (56.8)
Use of hydrocortisone, n (%)	74 (38.5)
Use of cortisone acetate, n (%)	118 (61.5)
Hydrocortisone equivalent doses, mg/day	20 (7.5-50.0)
Use of fludrocortisone, n (%)	189 (98.4)
Total fludrocortisone dose, mg/day	0.10 (0.03-0.30)
Women using DHEA, n (%)	16 (13.8)
Body mass index, kg/cm^2	24.4 (16.6-38.3)
Systolic blood pressure, mmHg	120 (84-169)
Diastolic blood pressure, mmHg	76 (50-95)
Hyperpigmentation, n (%)	100 (52.4)

Abbreviations: APS, autoimmune polyendocrine syndrome; DHEA, dehydroepiandrosterone; SD, standard deviation.

The highest recorded serum cortisol value (507 nmol/L) was found in a 68 year-old woman. At time of diagnosis 10 years earlier, she used estrogen replacement therapy. She was admitted due to weight

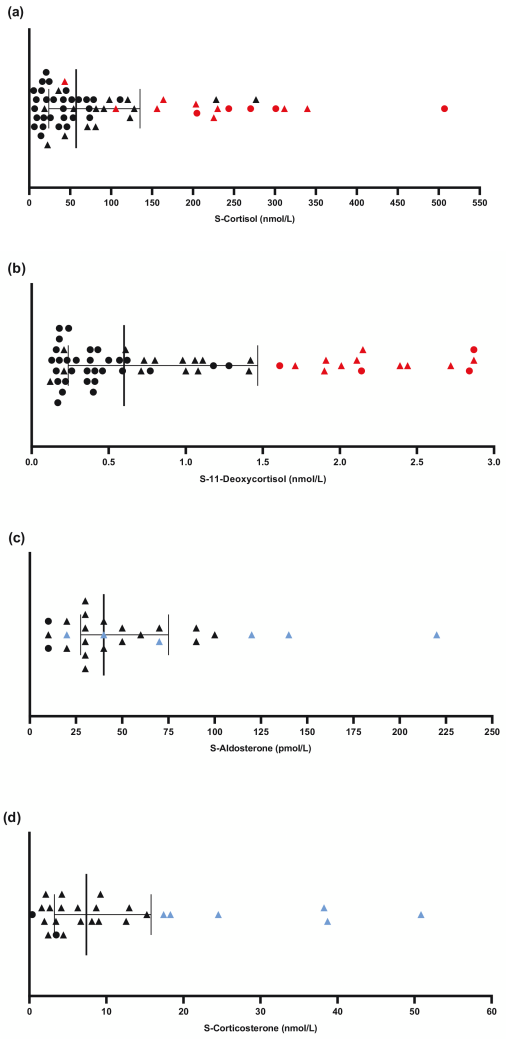


Figure 3. Stage 1: Corticosteroid levels in patients with residual glucocorticoid or mineralocorticoid production. The line marks median corticosteroid values and the whiskers the interquartile range. Triangles mark patients with both glucocorticoid and mineralocorticoid residual production. The patients with the highest quartile of 11-deoxycortisol and corticosterone values are marked in red and blue, respectively. (**A**) Serum cortisol at baseline (n = 58). (**B**) Serum 11-deoxycortisol values at baseline (n = 58). (**C**) Serum aldosterone values at baseline (n = 26). (**D**) Serum corticosterone values at baseline (n = 26).

loss, stomach pain, nausea and vomiting and had hyponatremia (124 mmol/L). Although serum cortisol was within normal range, plasma ACTH was elevated at 294 pmol/L, the maximal cortisol peak at cosyntropin test was suboptimal at 407 nmol/L, and the 21-hydroxylase autoantibody index was clearly elevated. Her symptoms were relieved after initiation of replacement therapy with hydrocortisone and FC. In addition, ACTH analyses, cosyntropin tests, and 21-hydroxylase autoantibody assays have been performed at several occasions after diagnosis and remained pathological. The patient reported several adrenal crises since receiving the diagnosis in 2010, including 1 incident last year due to gastrointestinal infection with vomiting and diarrhea.

Residual MC production. On group level, patients with MC residual production had shorter disease duration (median 5.5 [0.5-26.0] vs 13 [0-53] years; $P < 0.004$), lower FC replacement dosage (median 0.075 [0.050-0.120] vs 0.100 [0.028-0.300] mg; $P < 0.005$), higher PRC (median 179 [22-915] vs 47.5 [0.6-658.0] mU/L; $P < 0.001$), and higher levels of all but 5 steroids (18-oxo-cortisol, allo-tetrahydrocortisone, testosterone, epitestosterone, dihydrotestosterone; data not shown). For binary logistic regression on residual MC production, only PRC and disease duration significantly contributed to the model. The likelihood of residual MC production decreased with disease duration (OR 0.89; CI 95%, 0.82-0.96; $P < 0.003$) and slightly increased with higher PRC (OR 1.005; CI 95%, 1.002-1.008; $P < 0.001$. In sum, the regression model explained between 18.9% and 35.4% of the variance and correctly classified 90.8% of the cases ($X^2(9, N = 173) = 36.197$; $P < 0.001$).

The highest serum aldosterone level recorded (217 pmol/L) was found in a 23-year-old woman. Her plasma renin concentration exceeded the upper limit of detection (>500 mU/L). The patient also presented with a high cortisol (340 nmol/L) and did not use oral contraceptive pills or estrogen. Of note, the patient had experienced adrenal crisis twice since receiving the diagnosis in 2013 and suffers concomitant hypothyroidism, celiac disease, vitamin B12 deficiency, and previously Graves disease. At time of diagnosis, she fulfilled the diagnostic criteria for AD, with morning cortisol in the lower reference range, elevated ACTH level, and clearly elevated index of 21-hydroxylase autoantibodies.

Combined residual GC and MC production. Twenty-four patients had quantifiable levels of cortisol, 11-deoxycortisol, aldosterone, and corticosterone. They had significantly shorter disease duration (median 5.5 [0.5-26.0] vs 13.5 [0.0-53.0] years; $P < 0.002$), higher PRC (median 152 [22-915] vs 46 [1-658] mU/L; $P < 0.001$), and higher levels of all but 3 steroids (testosterone, epitestosterone, dihydrotestosterone; data not shown) compared with patients with no residual production. Individual patient data are presented in Table 3.

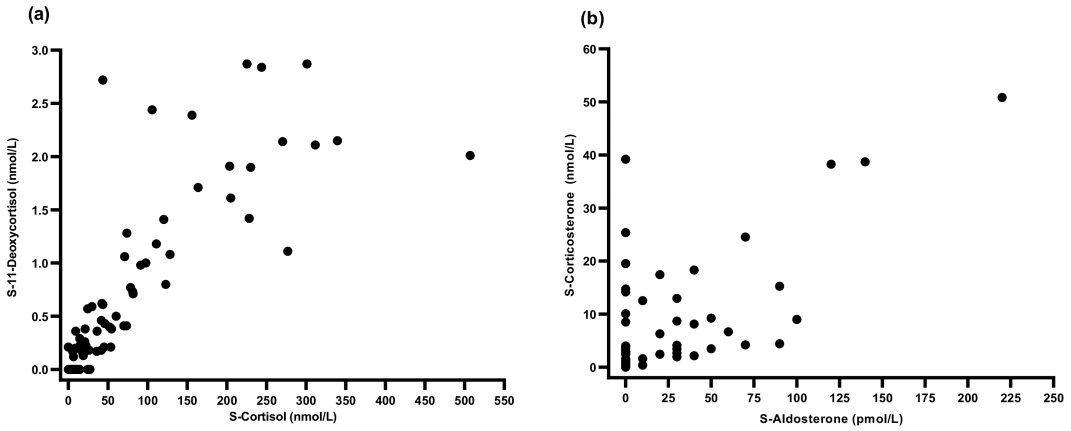


Figure 4. Correlation between corticosteroids. (**A**) Correlation between serum cortisol and 11-deoxycortisol ($P < 0.001$). (**B**) Correlation between serum aldosterone and corticosterone ($P < 0.001$).

Residual production and clinical characteristics. On group level, all routine laboratory values were within the reference intervals (Table 4). Patients with residual GC and/or MC production did not differ significantly from those without residual production regarding frequency of adrenal crises, number of infections the previous year, APS2, disease-related symptoms, hydrocortisone equivalent dosage, physical health, or HRQoL scores (AddiQoL and RAND-36) (Table 4).

Stage 2: Cosyntropin test

In total, 55 patients with residual GC production underwent the cosyntropin test. Three patients with quantifiable cortisol and 11-deoxycortisol at baseline declined. The screening results of residual GC production were verified in all but 5 patients. These patients were excluded from statistical analyses on cosyntropin test results. The remaining 50 patients reached a median peak cortisol of 75 [9-419] nmol/L (Fig. 5A), confirming the diagnosis of adrenal insufficiency. Higher serum cortisol levels at 0 minutes and lower plasma ACTH levels strongly correlated with peak cortisol ($r = 0.989$; $P < 0.001$, and $r = -0.487$; $P < 0.001$, respectively) (Figs. 5B and 5C).

The cosyntropin test was also performed in 2 patients with isolated residual MC production at screening, but upon testing aldosterone, it was only quantifiable for 1 of them. For this patient, aldosterone levels remained unchanged at 40 pmol/L throughout the test.

Twenty patients without quantifiable levels of cortisol and 11-deoxycortisol and/or aldosterone and corticosterone at stage 1 were included as controls. At cosyntropin testing, serum cortisol was barely quantifiable in 10 of the controls but remained unquantifiable in the other 10 controls. Two controls also had barely quantifiable levels of serum corticosterone, but none had quantifiable levels of serum 11-deoxycortisol or aldosterone.

Discussion

We found residual GC production in one-third of patients with established AAD, more common in men than in women. Patients with residual production had overall shorter disease duration, but several had a history of AAD lasting for decades. More than 1 of 7 patients had residual MC production. These were characterized by shorter disease duration, lower FC dosage, and higher plasma renin concentrations compared with those without residual MC production. No significant associations were found between residual corticosteroid production and a number of clinical parameters. To date, this is the largest study on residual production in AAD, conducted on a representative study cohort from 17 centers in 3 countries. We are confident that the diagnosis of AAD is correct in all included patients as we required documented presence of 21-hydroxylase antibodies and chronic need for GC replacement therapy for inclusion.

There is no established definition of residual corticosteroid production. LC-MS/MS enables measurement of minute quantities of cortisol and aldosterone; however, the clinical effect of very low cortisol and aldosterone concentrations is uncertain. We believe that merely evaluating serum cortisol levels would result in a falsely high prevalence of residual GC production, as up to half of the bioavailable cortisol stems from cortisone regenerated by 11-β-hydroxysteroid dehydrogenase type 1 (13). In addition, it is important to discriminate between endogenous and exogenous cortisol in these patients who use GC replacement therapy. This could

Table 3. Characteristicsof the 24 patients with combined glucocorticoid and mineralocorticoid residual production

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
S-F, nM	340	311	277	230	228	225	204	164	156	128	123	120	106	98	91	82	81	71	54	43	43	36	22	19
S-S, nM	2.2	2.1	1.1	1.9	1.4	2.9	1.9	1.7	2.4	1.1	0.8	1.4	2.4	1.0	1.0	0.7	0.7	1.1	0.2	0.6	2.7	0.2	0.2	0.1
S-Aldo, pM	217	69	135	121	56	25	14	15	86	104	39	39	15	92	53	28	29	51	66	31	24	38	10	33
S-CCN, nM	51	25	39	38	7	13	13	17	15	9	18	8	6	4	9	9	3	4	4	4	2	2	2	2
Sex, M or F	F	M	F	F	M	F	M	F	F	F	M	F	F	F	F	F	M	F	F	M	F	M	M	F
Age, years	23	63	32	43	62	51	18	47	40	59	30	53	55	51	49	53	18	45	66	26	23	38	36	42
DD, years	5	6	1	5	26	9	5	7	0.5	6	4	24	9	20	5	1	2	7	2	2	1	21	7	12
AC, yes or no	yes	yes	no	yes	yes	no	yes	yes	yes	no	yes	yes	no	no	yes	no	yes	no	yes	yes	no	no	yes	yes
HCeq., mg	20	25	20	20	20	10	28	30	30	20	25	20	15	10	17.5	20	30	20	20	40	30	30	30	25
BMI, kg/m^2	18.1	25.6	24.4	23.0	26.1	23.3	27.8	29	34.3	37.1	28.7	27.1	26.0	25.9	27.8	29.4	20.8	22	21.0	21.2	22.0	26.5	22.3	18.8
AddiQoL score	66	89	72	102	94	95	90	105	73	117	86	80	93	95	80	96	102	101	93	99	83	91	*	91
P-ACTH, pmol/L	63	32	26	70	34	68	39	210	62	237	82	67	120	134	88	259	224	225	31	278	125	43	278	175
PRC, mU/L	500	187	146	302	48	308	76	325	122	124	179	22	*	465	61	337	77	107	22	152	350	214	81	915

Abbreviations: AAD, autoimmune Addison disease; AC, adrenal crisis ever; AddiQoL, AAD-specific questionnaire; BMI, body mass index; DD, disease duration; F, female; HCeq, hydrocortisone-equivalent dose; M, male; P-ACTH, plasma adrenocorticotropic hormone; PRC, plasma renin concentration; S-Aldo, serum aldosterone; S-CCN, serum corticosterone S-F, serum cortisol, S-S, serum 11-deoxycortisol.
* Not obtained.

in part be avoided by having patients abstain from GC replacement therapy for a longer period of time but would put them at risk of developing an adrenal crisis. Concerning residual MC production, we are not aware of any bidirectional pathways in aldosterone metabolism. Furthermore, FC is a synthetic MC and does not interfere with aldosterone measurements on LC-MS/MS (14). In the present study, patients were asked to abstain from GC and MC replacement therapy for at least 18 and 24 hours, respectively, before sample collection. To further ensure that the measured hormones indeed represented de novo synthesis of corticosteroids, we chose to include precursors for the definitions of residual GC and MC production. Importantly, the enzymes involved in conversion of the precursors to the active substances are considered unidirectional (15), precluding any synthesis of precursors from cortisol or aldosterone. This was well illustrated by 1 of the study participants who had a serum cortisol level of 797 nmol/L but no quantifiable 11-deoxycortisol. Later, it become known that she had taken her morning dose of cortisone acetate before the blood sampling but had forgotten to mention it. The patient was therefore excluded. In patients with residual production, we found that median and range values of 11-deoxycortisol and corticosterone corresponded with values found in healthy controls (16), suggesting that these are suitable as biomarkers of residual production.

We were surprised to detect a clear overweight of men with residual GC production, despite women constituting the majority of our study cohort. This may be due to sex-related disparities in immunology as well as susceptibility to autoimmune disease (17). It has been suggested that inherent sex differences in adrenal gland tissue renewal could be involved (18). Indeed, in mice, the turnover rate for adrenocortical tissue is 3 times higher in females compared with males, and capsular stem cells only contribute to tissue renewal in females, not in males (18). Whether these findings are relevant for humans is not known, and highlights the need for future studies to explore the impact of sex on the trajectory of autoimmune adrenalitis.

As expected, the patients with GC and/or MC residual production had shorter median disease duration. However, there was a wide range in disease duration among the patients with residual production, extending up to 26 years for MC and 44 years for GC residual production, arguing against the common assumption that AAD inevitably leads to loss of all adrenal corticosteroid production. Concurrently, it raises questions of how and why the intensity and extent of the autoimmune attack seem to differ between individuals.

Regarding steroid replacement therapy, we found significantly lower dosages of FC in patients with residual

Table 4. Differences in patient characteristics between patients with and without residual glucocorticoid production and in patients with combined glucocorticoid and mineralocorticoid production compared with patients with no residual production.

	N (%) or Median (minimum, maximum) or Mean (±SD)					
Variable	**GC+**	**GC–**	***P***	**GC+, MC+**	**GC–, MC–**	***P***
No. females (%)	25 (43.1)	91 (67.9)	0.001[a]	16 (66.7)	89 (67.4)	1.000
Age, years	46.2 ± 14.8	49.2 ± 12.3	0.142	42.6 ± 14.4	49.3 ± 12.3	0.020
DD, years	6 (0-44)	13 (0-53)	0.001[a]	5.5 (0.5-26)	13.5 (0.0-53.0)	0.002[a]
Age at diagnosis, years	36 (12-64)	31.5 (11-63)	0.202	33.5 (13-64)	31 (11-63)	0.673
Adrenal crisis ever, n (%)	38 (65.6)	98 (73.7)	0.252	15 (62.5)	96 (73.3)	0.406
Adrenal crisis at diagnosis, no (%)	34 (58.6)	79 (59.4)	0.920	13 (54.2)	78 (59.5)	0.790
Adrenal crisis last year, n (%)	11 (19.0)	19 (14.3)	0.414	5 (20.8)	19 (14.5)	0.630
Infectious illness last year, n (%)	22 (37.9)	49 (37.1)	0.915	7 (29.2)	49 (37.7)	5.710
CVD, no. (%)	0 (0)	2 (1.5)	0.350	0 (0)	2 (1.5)	1.000
Osteoporosis, n (%)	4 (6.9)	12 (9.0)	0.626	0 (0)	12 (9.2)	0.259
APS2, no. (%)	29 (50)	80 (59.7)	0.277	14 (58.3)	78 (59.1)	0.945
Salt cravings, n (%)	14 (24.1)	33 (24.6)	0.942	8 (33.3)	32 (24.2)	0.494
Orthostatic hypotension, n (%)	14 (24.1)	18 (13.4)	0.068	8 (33.3)	18 (13.6)	0.037
Fatigue, n (%)	22 (37.9)	54 (40.3)	0.758	7 (29.2)	54 (40.5)	0.391
Loss of appetite, n (%)	6 (10.3)	6 (4.5)	0.123	2 (8.3)	6 (4.5)	0.786
GI-symptoms, n (%)	14 (24.1)	25 (18.7)	0.386	5 (20.8)	25 (18.9)	1.000
Muscle/ joint pain, n (%)	17 (29.3)	33 (24.6)	0.497	4 (16.7)	33 (25.0)	0.534
Sleeping disturbances, n (%)	20 (34.5)	36 (26.9)	0.286	11 (45.8)	36 (27.3)	0.114
Nausea, n (%)	4 (6.9)	10 (7.5)	0.890	4 (16.7)	10 (7.6)	0.296
BMI, kg/m^2	25.1 (18.1-37.1)	24.1 (16.6-38.3)	0.257	25.8 (18.1-37.1)	24.1 (16.6-38.3)	0.512
SBP, mmHg	120.5 (90-150)	120 (84-169)	0.019	120 (90-150)	120 (84-169)	0.356
DPB, mmHg	76 (55-93)	76 (50-95)	0.537	75 (55-90)	76 (50-95)	0.737
Hyperpigmentation, n (%)	29 (50.0)	71 (53.4)	0.667	11 (45.8)	70 (53.4)	0.643
HCeq, mg/day	20 (10-50)	20 (7.4-40)	0.875	20 (10-40)	20 (7.5-40)	0.375
HCeq, mg/kg/day	0.31 (0.14-0.58)	0.32 (0.12-0.78)	0.179	0.31 (0.14-0.55)	0.32 (0.03-0.3)	0.484
HCeq, mg/m^2/day	7.4 (3.3-13.3)	7.7 (2.8-15.4)	0.179	7.9 (3.3-12.0)	7.8 (2.8-15.4)	0.839
FC, mg/ day	0.10 (0.05-0.20)	0.10 (0.03-0.30)	0.156	0.10 (0.05-0.12)	0.10 (0.03-0.30)	0.014
RAND-36 PF	95 (55-100)	95 (25-100)	0.710	95 (80-100)	95 (35-100)	0.395
RAND-36 RP	100 (0-100)	100 (0-100)	0.444	100 (0-100)	100 (0-100)	0.087
RAND-36 BP	74 (22-100)	84 (12-100)	0.835	84 (22-100)	83 (12-100)	0.363
RAND-36 GH	67 (17-100)	67 (5-95)	0.879	67 (20-97)	67 (10-100)	0.718
RAND-36 VT	65 (5-100)	60 (5-95)	0.407	65 (5-100)	60 (5-95)	0.198
RAND-36 SF	87.5 (25-100)	87.5 (12.5-100)	0.991	87.5 (25-100)	87.5 (12.5-100)	0.653
RAND-36 RE	100 (0-100)	100 (0-100)	0.374	100 (0-100)	100 (0-100)	0.605
RAND-36 MH	80 (36-100)	84 (44-100)	0.534	76 (36-100)	84 (55-100)	0.156
AddiQol-30	90.9 ± 12.7	89.6 ± 10.3	0.476	91 ± 11.7	89.5 ± 10.3	0.5230
Hb, g/dL	14.5 ± 1.2	13.8 ± 1.1	0.001[a]	14.6 ± 1.2	13.8 ± 1.1	0.004[a]
HbA_{1C}, mmol/mol	35 (28-53)	35 (24-43)	0.657	35 (28-50)	35 (24-43)	0.806
S-TSH, mIE/L	2.6 (0.05-12.7)	2.55 (0.01-13.2)	0.977	1.8 (0.06-7.0)	2.6 (0.01-13.2)	0.140
S-fT_4, pmol/L	15.0 (10.6-23.0)	15.0 (10.6-25.0)	0.983	16.2 (11-23)	15.0 (10.6-25.0)	0.262
S-cobalamin, pmol/L	368 (174-753)	372 (140-1476)	0.964	379 (210-605)	372 (140-1476)	0.490
S-ferritin µg/L	116 (15-446)	101 (6-621)	0.394	91 (15-297)	102 (6-621)	0.416
S-creatinine, µmol/L	77 (60-150)	73 (39-116)	0.006[a]	75 (60-96)	73 (39-116)	0.405

Table 4. Continued

Variable	N (%) or Median (minimum, maximum) or Mean (±SD)					
	GC+	GC–	*P*	GC+, MC+	GC–, MC–	*P*
S-sodium, mmol/L	139 (131-145)	140 (131-148)	0.237	138 (136-142)	140 (131-148)	0.005[a]
S-potassium, mmol/L	4.1 (3.5-4.9)	3.9 (3.0-5.1)	0.008[a]	4.2 (3.5-4.6)	3.9 (3.0-5.1)	0.036
S-cholesterol, mmol/L	5.1 ± 1.0	5.1 ± 0.9	0.630	5.1 ± 1.2	5.1 ± 1.0	0.827
S-HDL-C, mmol/L	1.4 (0.1-2.2)	1.7 (0.6-2.7)	0.001[a]	1.5 (1.1-2.2)	1.7 (0.6-2.7)	0.224
S-LDL-C, mmol/L	3.2 ± 1.0	3.1 ± 0.8	0.406	3.3 ± 1.2	3.1 ± 0.8	0.292
S-triglycerides, mmol/L	1.3 (0.4-5.8)	1.3 (0.1-9.7)	0.609	1.2 (0.5-3.2)	1.3 (0.1-9.7)	0.702
PRC, mU/L	75.7 (0.7-915.0)	49.0 (0.6-658.0)	0.055	152 (22-915)	46 (1-658)	<0.001[a]
P-ACTH, pmol/L	123 (26-278)	147 (1-278)	0.123	85 (26-278)	147 (1-278)	0.087

Abbreviations: APS2, autoimmune polyendocrine syndrome type 2; BMI, body mass index; BP, bodily pain; CVD, cardiovascular disease; DBP, diastolic blood pressure; DD; disease duration; FC, fludrocortisone dosage; fT_4, free thyroxine; GC+, residual glucocorticoid production; GC–, no residual glucocorticoid production; GH, general health; GI, gastrointestinal; Hb, hemoglobin; HbA_{1c}, glycated hemoglobin; HCeq, hydrocortisone equivalent dosage; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MC+, residual mineralocorticoid production; MC–, no residual mineralocorticoid production; MH, general mental health; P-ACTH, plasma adrenocorticotropic hormone; PF, physical functioning; PRC, plasma renin concentration; RAND, health survey; RE, role limitations caused by emotional problem; RP, role limitations caused by physical health problems; S-, serum; SBP, systolic blood pressure; SD, standard deviation; SF, social functioning; TSH, thyroid-stimulating hormone; VT, vitality.
[a]Statistically significant at 0.01 level.

MC production. This could, of course, be due to lower replacement needs. As these participants also had higher levels of plasma renin concentration, one could speculate if greater renin exposure via an activated renin-angiotensin-aldosterone system may stimulate MC production in remnants of the zona glomerulosa. We did not find any association between residual corticosteroid production and hydrocortisone-equivalent dosages. This might be masked by the fact that GC receptor polymorphisms influence the GC replacement dose (19). In addition, there is currently no available biomarker to guide optimization of GC replacement treatment. When evaluating FC dosages, the physician is aided by the patient's blood pressure, electrolyte levels, and plasma renin concentration (20). For GC therapy, however, surveillance relies upon more vague clinical signs and the patient's subjective health status (21). Therefore, we cannot rule out that patients with residual GC production receive unnecessarily high GC dosages. If true, residual production could put patients at risk of deleterious health effects due to GC excess, including cardiovascular disease (22), infections (23), and premature death (24). Whether residual production enables safe dose reductions should be explored in further studies.

Of note, we found no differences in frequency of adrenal crises, infections, APS2, disease-related symptoms, physical health, or HRQoL in patients with and without residual production of adrenal corticosteroids. An obvious explanation is, of course, that no such links exist. Yet, as with any exploratory study, we must acknowledge that our chosen methods may not have been ideal for evaluating the clinical significance of residual GC and MC production. Furthermore, quantifiable levels of adrenal corticosteroids may not represent clinically significant values. Inaccuracies due to recall bias must also be considered, especially for the frequencies of adrenal crises and infections that were self-reported by the patients.

In line with previous studies, none of the patients in the current study had a normal response to the cosyntropin test (2, 4, 25, 26). Still, patients with higher cortisol levels before injection of cosyntropin reached significantly higher peak cortisol, suggesting a greater stimulatory potential. Indeed, in attempts to regenerate adrenocortical function in AAD by rituximab and/or tetracosactide, lasting recovery has only been reported in 2 patients with cosyntropin-stimulated peak cortisol of 219 and 235 nmol/L before treatment initiation (4, 25, 26).

Unfortunately, our study design did not allow us to answer the compelling questions on the nature and origin of residual production in AAD. In order to investigate possible heterogeneity in disease development and adrenal plasticity, we call for a prospective study including newly diagnosed

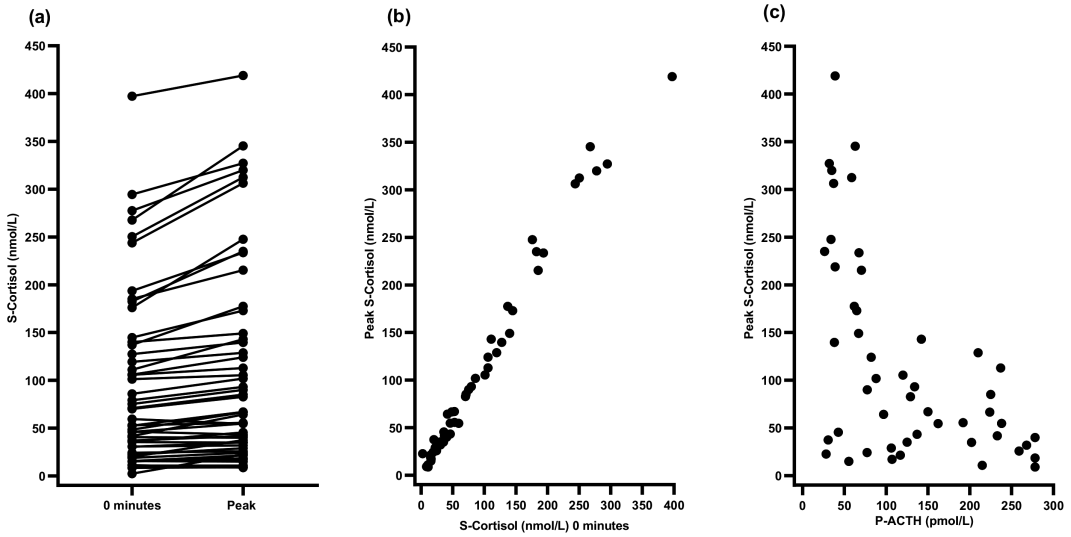


Figure 5. Cosyntropin testing. (**A**) Change in serum cortisol before (0 minutes) intravenous 250 µg cosyntropin to peak serum cortisol after 30 or 60 minutes. (**B**) Correlation between serum cortisol before (0 minutes) intravenous 250 µg cosyntropin and peak serum cortisol at 30 or 60 minutes ($P < 0.001$). (**C**) Correlation between plasma ACTH before (0 minutes) intravenous 250 µg cosyntropin and peak serum cortisol 30 or 60 minutes ($P < 0.001$). ACTH, adrenocorticotropic hormone.

individuals to be assessed at baseline and followed annually. Such a study could ascertain whether certain AAD subpopulations are more resistant to immune-mediated destruction, perhaps by harboring other human leukocyte antigen genotypes than patients without residual production, or if the intensity of autoimmune destruction may vary over time allowing regeneration of steroid-producing cells.

In our opinion, remnants of functional adrenocortical tissue are the most probable origin of residual production. We suggest 2 possible mechanisms: Either areas in the adrenal cortex have been spared from autoimmune attack or adrenocortical cells could be replenished by differentiation of subcapsular stem cells (27). Both are in line with observations in autoimmune type 1 diabetes where pancreatic infiltration of immune cells is not always uniform but may be patchy and leave subsets of pancreatic islets unaffected (28). Indeed, recent reports suggest that residual beta cell capacity may be present in one-third of patients with longstanding type 1 diabetes (28).

An alternative explanation is extra-adrenal production. The observed male preponderance in residual GC production opens for a tantalizing link to hormone-producing testicular adrenal rest tumors (TARTs), as seen in approximately 40% of men with congenital adrenal hyperplasia (29). However, a recent ultrasonographic screening of 14 men with Addison disease could not detect any cases of TART (30). Moreover, if TARTs indeed were the true sources of residual production, there would still be the question on how cortisol-producing cells evade the autoimmune attack, as the Leydig cells are located outside the blood-testis barrier (31). In conclusion, one-third of patients with autoimmune Addison disease still produce GCs and MCs even years after the diagnosis, more commonly observed in men in our cohort. These findings challenge our current understanding of the natural course of the disease.

Acknowledgements

We thank Mona Eliassen, Nina Jensen (Haukeland University Hospital), Lillian Skumsnes (Haugesund Hospital), Hanne Høivik Bjørkås and Elise Turkerud Søby (Innlandet Hospital Trust), Maria Wärn (Karolinska University Hospital), Christina Dahlgren (University Hospital Linköping), Katarina Iselid (Umeå University Hospital), Anette Nilsson (Central Hospital Karlstad), Kari Irene Abelsen (Oslo University Hospital), Anne Breikert (University Hospital Örebro), and Britta Bauer (Endocrinology in Charlottenburg, Berlin, Germany) for good patient care and collection of blood samples. We thank Åsa Hallgren (Karolinska Institutet) and Øyvind Skadberg (Stavanger University Hospital) for logistics support. We also thank Lars Breivik and Elisabeth Tombra Halvorsen (Endocrinological Research Laboratory, Department of Clinical Science, University of Bergen) for helping organizing the study and handling blood samples. Great thanks to Nina Henne and Nebeyaet Selemon Gebreslase (Core Facility for Metabolomics, Department of Clinical Science, University of Bergen) for analyzing samples on LC-MS/MS, and to Anders Engeland (Department of Global Public Health and Primary Care, University of Bergen) for statistical counseling. Lastly, we thank all the patients who participated and made this study possible. This work was supported with grants from the The

Research Council of Norway, The Novo Nordisk Foundation, The Internal Medicine Association of Norway, and The Legate of Dr. Nils Henrichsen and Wife Anna Henrichsen. S.B. grant the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet.

***Financial Support*:** The Research Council of Norway, the Novo Nordisk Foundation, the Internal Medicine Association of Norway, and the Legate of Dr. Nils Henrichsen and Wife Anna Henrichsen provided financial support.

Clinical Trial Information: **CinicalTrials.gov registration number:** NCT03793114 (November 06, 2018).

Additional Information

Correspondence and Reprint Requests: Marianne Øksnes, University of Bergen, Klinisk Institutt 2, Laboratoriebygget, 8. et., Jonas Lies vei 91B, 5021 Bergen, Norway, E-mail: Marianne.Oksnes@uib.no.

***Disclosure Statement*:** The authors have nothing to disclose.

Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Eisenbarth GS, Gottlieb PA. Autoimmune polyendocrine syndromes. *N Engl J Med*. 2004;**350**(20):2068-2079.
2. Smans LC, Zelissen PM. Does recovery of adrenal function occur in patients with autoimmune Addison's disease? *Clin Endocrinol (Oxf)*. 2011;**74**(4):434-437.
3. Vulto A, Bergthorsdottir R, van Faassen M, Kema IP, Johannsson G, van Beek AP. Residual endogenous corticosteroid production in patients with adrenal insufficiency. *Clin Endocrinol (Oxf)*. 2019;**91**(3):383-390.
4. Napier C, Gan EH, Mitchell AL, et al. Residual adrenal function in autoimmune Addison's disease - effect of dual therapy with rituximab and depot tetracosactide. *J Clin Endocrinol Metab*. 2020;**105**(4):e1250-e1259.
5. Dalin F, Nordling Eriksson G, Dahlqvist P, et al. Clinical and immunological characteristics of autoimmune Addison's disease: a nationwide Swedish multicenter study. *J Clin Endocrinol Metabo*. 2017;**102**(2):379-389.
6. Hahner S, Loeffler M, Bleicken B, et al. Epidemiology of adrenal crisis in chronic adrenal insufficiency: the need for new prevention strategies. *Eur J Endocrinol*. 2010;**162**(3):597-602.
7. Løvås K, Husebye ES. High prevalence and increasing incidence of Addison's disease in western Norway. *Clin Endocrinol (Oxf)*. 2002;**56**(6):787-791.
8. Erichsen MM, Løvås K, Skinningsrud B, et al. Clinical, immunological, and genetic features of autoimmune primary adrenal insufficiency: observations from a Norwegian registry. *J Clin Endocrinol Metab*. 2009;**94**(12):4882-4890.
9. Ueland GÅ, Methlie P, Øksnes M, et al. The short cosyntropin test revisited: new normal reference range using LC-MS/MS. *J Clin Endocrinol Metab*. 2018;**103**(4):1696-1703.
10. Methlie P, Hustad SS, Kellmann R, et al. Multisteroid LC-MS/MS assay for glucocorticoids and androgens, and its application in Addison's disease. *Endocr Connect*. 2013;**2**(3):125-136.
11. Hays RD, Sherbourne CD, Mazel RM. The RAND 36-Item Health Survey 1.0. *Health Econ*. 1993;**2**(3):217-227.
12. Øksnes M, Bensing S, Hulting AL, et al. Quality of life in European patients with Addison's disease: validity of the disease-specific questionnaire AddiQoL. *J Clin Endocrinol Metab*. 2012;**97**(2):568-576.
13. Walker BR, Andrew R. Tissue production of cortisol by 11beta-hydroxysteroid dehydrogenase type 1 and metabolic disease. *Ann N Y Acad Sci*. 2006;**1083**:165-184.
14. Pussard E, Travers S, Bouvattier C, et al. Urinary steroidomic profiles by LC-MS/MS to monitor classic 21-hydroxylase deficiency. *J Steroid Biochem Mol Biol*. 2020;**198**:105553.
15. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011;**32**(1):81-151.
16. Eisenhofer G, Peitzsch M, Kaden D, et al. Reference intervals for plasma concentrations of adrenal steroids measured by LC-MS/MS: impact of gender, age, oral contraceptives, body mass index and blood pressure status. *Clin Chim Acta*. 2017;**470**:115-124.
17. Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol*. 2016;**16**(10):626-638.
18. Grabek A, Dolfi B, Klein B, Jian-Motamedi F, Chaboissier MC, Schedl A. The adult adrenal cortex undergoes rapid tissue renewal in a sex-specific manner. *Cell Stem Cell*. 2019;**25**(2):290-296.e2.
19. Koetz KR, van Rossum EF, Ventz M, Diederich S, Quinkler M. BclI polymorphism of the glucocorticoid receptor gene is associated with increased bone resorption in patients on glucocorticoid replacement therapy. *Clin Endocrinol (Oxf)*. 2013;**78**(6):831-837.
20. Pofi R, Prete A, Thornton-Jones V, et al. Plasma renin measurements are unrelated to mineralocorticoid replacement dose in patients with primary adrenal insufficiency. *J Clin Endocrinol Metab*. 2020;**105**(1):dgz055.
21. Husebye ES, Allolio B, Arlt W, et al. Consensus statement on the diagnosis, treatment and follow-up of patients with primary adrenal insufficiency. *J Intern Med*. 2014;**275**(2):104-115.
22. Skov J, Sundström A, Ludvigsson JF, Kämpe O, Bensing S. Sex-Specific risk of cardiovascular disease in autoimmune Addison disease-a population-based cohort study. *J Clin Endocrinol Metab*. 2019;**104**(6):2031-2040.
23. Tresoldi AS, Sumilo D, Perrins M, et al. Increased infection risk in Addison's disease and congenital adrenal hyperplasia. *J Clin Endocrinol Metab*. 2020;**105**(2):418-429.
24. Quinkler M, Ekman B, Zhang P, Isidori AM, Murray RD; EU-AIR Investigators. Mortality data from the European Adrenal Insufficiency Registry-patient characterization and associations. *Clin Endocrinol (Oxf)*. 2018;**89**(1):30-35.
25. Gan EH, MacArthur K, Mitchell AL, et al. Residual adrenal function in autoimmune Addison's disease: improvement after tetracosactide (ACTH1-24) treatment. *J Clin Endocrinol Metab*. 2014;**99**(1):111-118.
26. Pearce SH, Mitchell AL, Bennett S, et al. Adrenal steroidogenesis after B lymphocyte depletion therapy in new-onset Addison's disease. *J Clin Endocrinol Metab*. 2012;**97**(10):E1927-E1932.
27. Gan EH, Robson W, Murphy P, Pickard R, Pearce S, Oldershaw R. Isolation of a multipotent mesenchymal stem cell-like population from human adrenal cortex. *Endocr Connect*. 2018;**7**(5):617-629.
28. Oram RA, Sims EK, Evans-Molina C. Beta cells in type 1 diabetes: mass and function; sleeping or dead? *Diabetologia*. 2019;**62**(4):567-577.
29. Engels M, Span PN, van Herwaarden AE, Sweep FCGJ, Stikkelbroeck NMML, Claahsen-van der Grinten HL. Testicular adrenal rest tumors: current insights on prevalence, characteristics, origin, and treatment. *Endocr Rev*. 2019;**40**(4):973-987.
30. Verhees MJM, Kamphuis-Van Ulzen K, Hermus A, Stikkelbroeck N, Mooij CF, Claahsen-van der Grinten HL. Re: testicular adrenal rest tumors in boys and young adults with congenital adrenal hyperplasia: Kim M. S. Goodarzian F., Keenan M. F., Geffner M. E., Koppin C. M., De Filippo R. E., and Kokorowski P. J., J Urol 2017;197:931–936. The Journal of urology. 2018;199(5):1357-1358.
31. Mruk DD, Cheng CY. The mammalian blood-testis barrier: its biology and regulation. *Endocr Rev*. 2015;**36**(5):564-591.

Altered biomarkers for cardiovascular disease and inflammation in autoimmune Addison's disease

Åse Bjorvatn Sævik[1,2], Grethe Ueland[1,2,3], Anna-Karin Åkerman[4,5], Paal Methlie[1,2,3], Marcus Quinkler[6], Anders Palmstrøm Jørgensen[7], Charlotte Höybye[4,8], Aleksandra W. J. Debowska[9], Bjørn Gunnar Nedrebø[10], Anne Lise Dahle[10], Siri Carlsen[11], Aneta Tomkowicz[12], Stina Therese Sollid[13], Ingrid Nermoen[14], Kaja Grønning[14], Per Dahlqvist[15], Guri Grimnes[16,17], Jakob Skov[4], Trine Finnes[18], Susanna F Valland[18], Jeanette Wahlberg[19]; Synnøve Emblem Holte[20], Olle Kämpe[2,8,21], Sophie Bensing[4,8], Eystein Sverre Husebye[1,2,5,21], Marianne Øksnes[1,2,5,21]

Affiliations:

[1]Department of Clinical Science, University of Bergen, Norway; [2]K.G. Jebsen center for Autoimmune Disorders, University of Bergen, Bergen, Norway; [3]Department of Medicine, Örebro University Hospital, Örebro, Sweden; [4]Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; [5]Department of Medicine, Haukeland University Hospital, Bergen, Norway; [6]Endocrinology in Charlottenburg, Berlin, Germany; [7]Department of Endocrinology, Oslo University Hospital, Oslo, Norway; [8]Department of Endocrinology, Karolinska University Hospital, Stockholm, Sweden; [9]Department of Medicine, Vestfold Hospital Trust, Tønsberg, Norway; [10]Department of Internal Medicine, Haugesund Hospital, Haugesund, Norway; [11] Department of Endocrinology, Stavanger University Hospital, Stavanger,

Norway; [12]Department of Medicine, Sørlandet Hospital, Kristiansand, Norway; [13]Department of Medicine, Drammen Hospital, Vestre Viken Health Trust, Drammen, Norway; [14]Department of Endocrinology, Akershus University Hospital, Lørenskog, Norway; [15]Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden; [16]Division of Internal Medicine, University Hospital of North Norway, Tromsø, Norway; [17]Tromsø Endocrine Research Group, Department of Clinical Medicine, UiT the Arctic University of Norway, Tromsø, Norway; [18]Section of Endocrinology, Innlandet Hospital Trust, Hamar, Norway; [19]Department of Endocrinology and Department of Health, Medicine and Caring Sciences, Linköping University, Linköping, Sweden; [20]Department of Medicine, Sørlandet Hospital, Arendal, Norway; [21]Department of Medicine (Solna), Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden

Corresponding author:

Åse Bjorvatn Sævik

Department of Clinical Science, University of Bergen

Laboratory building, 8th floor

Jonas Lies vei 91B

5021 Bergen, Norway.

Email: aase.saevik@uib.no.

Short title: Biomarkers of CVD and inflammation in AAD

Keywords: autoimmunity, primary adrenal insufficiency, PAI, biomarkers, proteomics, cardiovascular disease, CVD

No. words: 3276

Abstract

Objective: Increased prevalence of cardiovascular disease and cardiovascular risk factors has been reported in autoimmune Addison's disease (AAD), but results are conflicting and the pathomechanisms poorly understood.

Design: Cross-sectional case-control study.

Methods: We compared serum levels of 177 cardiovascular and inflammatory biomarkers in 43 patients with AAD and 43 matched controls, overall and stratified for sex. Next, we correlated levels of significant biomarkers to frequency of adrenal crises and quality of life (QoL) by AddiQoL-30. Finally, we investigated changes in biomarker levels following high to very high ACTH exposure in patients without residual adrenocortical function (RAF).

Results: Nineteen (11%) biomarkers significantly differed between patients with AAD and controls, all but one (ST1A1) were higher in AAD. The greatest difference was noted for FGF21 (0.80 NPX, P=0.004). Eight biomarkers were significantly higher in female patients compared with controls (IL6, MCP1, GAL9, SPON2, DR4, RAGE, TNFRSF9, PGF), but none differed between male patients and controls. Levels of RAGE correlated with frequency of adrenal crises (r=0.415, P=0.006) and AddiQoL-30 scores (r=-0.347, P= 0.028). PDL2 and leptin significantly declined 60 minutes after injection of ACTH in AAD without RAF (-0.15 NPX, P=0.0001 and -0.25 NPX, P=0.0003, respectively).

Conclusions: We show that cardiovascular and inflammatory biomarkers are altered in AAD compared with controls, particularly in women. RAGE might be a marker of disease severity in AAD, associated with more adrenal crises and reduced QoL. Very high ACTH levels reduce PDL2 and leptin in a glucocorticoid-independent manner, although the overall effect on biomarker profiles was small.

Significance statement

Cardiovascular health seems to be impaired in AAD, but which patients carry the highest risk and why is not fully known. We show that biomarkers of CVD and inflammation are altered in AAD, with higher levels of 18 biomarkers and lower levels of one biomarker compared with controls. Eight biomarkers differed between female patients and controls but none between male patients and controls. Higher RAGE levels were linked to more adrenal crisis and lower QoL. Very high ACTH exposure reduced PDL2 and leptin levels in a glucocorticoid-independent manner but had otherwise little impact on biomarker profiles. Our results indicate sex-specific differences in CVD risk and RAGE as a possible marker of disease severity in AAD, to be explored further.

Introduction

Glucocorticoid (GC) and mineralocorticoid (MC) replacement therapy do not fully restore health in autoimmune Addison's disease (AAD), as patients continue to suffer reduced quality of life (QoL), risk of fatal adrenal crisis, and cardiovascular disease (CVD) (1). Swedish population-based studies have demonstrated higher prescription rates for CVD medications in patients with AAD, and increased risk of ischemic heart disease, especially in women (2, 3). In contrast, another Swedish study found obesity and hypertension to be less common in patients with AAD compared to population controls, and no difference in the frequency of other important CVD risk factors such as dyslipidemia or type 2 diabetes (4). These inconsistencies emphasize the need for a better understanding of what drives CVD risk in AAD and which subgroups of patients are most vulnerable.

Current theory highlights the inability of conventional GC replacement to replicate the circadian and ultradian rhythmicity of cortisol for unfavorable cardiovascular outcomes in adrenal insufficiency (5, 6), but factors beyond unphysiological GC replacement likely play important roles as well (7-10).

Rarely investigated in AAD, the autoimmune etiology could possibly contribute to CVD risk. A recent population-based study of CVD incidence rates in 19 common autoimmune diseases found each autoimmune disease to be independently associated with CVD, with an average elevated risk corresponding to a 20 mmHg rise in systolic blood pressure or the presence of type 2 diabetes (8). On group-level, patients with AAD had the second highest incidence rate of CVD among all studied autoimmune diseases. Any cardiovascular side effects of medications used, such as GCs, were not considered. Still, CVD risk increased with the number of concomitant autoimmune diseases, indicating a pattern of heightened risk common for autoimmune diseases rather than the individual diseases *per sé* (8).

We recently demonstrated that a subgroup of patients with AAD have residual adrenocortical function (RAF) (11), but any immunological differences between patients with and without RAF have not been explored. For AAD in general, elevated levels of cytokines and aberrant immune cell function of both innate and adaptive immunity have been reported even linked to persistent symptoms (e.g. reduced quality of life) and increased susceptibility to infections (12-17). However, most studies have been restricted to selected immune cells and molecules, calling for proteomic approaches to give a broader understanding of the proinflammatory state in AAD.

In preclinical AAD, ACTH levels increase to compensate for the progressive loss of adrenocortical cells and remain elevated due to shortcomings of conventional GC replacement (18). By promiscuous binding to the full range of melanocortin receptors (MC1-5R), ACTH might in theory modulate CVD risk and inflammation in AAD (19). But studies on extra-adrenal effects of elevated ACTH *in vivo* are typically hampered by difficulties in distinguishing GC-mediated and GC-independent effects.

Here, we mapped 177 cardiovascular and inflammatory biomarkers in patients with AAD compared with healthy controls. Second, we explored biomarker associations to the frequency of adrenal crises and quality of life (QoL), as well as any glucocorticoid-independent impact of very high ACTH exposure on biomarker profiles in patients without RAF.

Methods

Patients and samples

Using a cross-sectional study design, we included 43 patients with AAD, of whom 23 had confirmed RAF. The inclusion criteria have previously been reported in detail (11), and noted clinical characteristics

included the number of adrenal crises the past year and QoL by the disease-specific AddiQoL-30 questionnaire. In addition, we included 43 healthy controls matched for sex, age (in decade), and BMI (± 1 kg/m^2) from a previous clinical study (20). Six female patients with AAD and two female controls used oral contraceptive pills. Five female patients also used DHEA replacement (12-25 mg), but this was paused for at least one week prior to blood sampling. One male patient had a history of statin use.

Morning ACTH stimulation tests were performed in both patients and controls with intravenous injection of synthetic $ACTH_{1-24}$ (250 µg Synacthen®). Patients with AAD had abstained from any glucocorticoid and mineralocorticoid replacement for at least 18 and 24 hours, respectively. All participants were non-fasting as an extra safety measure for patients upon GC withdrawal. We analyzed serum samples collected before and 60 minutes after ACTH injection in both patients and controls. All samples were stored at -80°C before analysis.

Analysis of cardiovascular and inflammatory biomarkers

We employed the validated Cardiovascular II (CVD II) and Inflammation panels by Olink (Uppsala, Sweden), which contain 177 proteomic markers of cardiovascular and inflammatory physiology and disease. A complete list of biomarkers with coefficient of variance (CV%), lower limit of detection (LOD) and biomarker synonyms is included in Supplementary Table 1. The biomarkers were analyzed by proximity extension assay (PEA) technology, which combines dual-recognition immunoassay and quantitative PCR, yielding improved assay specificity and multiplexing capacity (21, 22). The results are given as normalized protein expression (NPX), which is an arbitrary unit on a Log2 scale calculated from normalized Ct values. Thus, NPX may only be used for relative quantification, and an increase of one unit (1 NPX) represents a doubling in protein concentration (23, 24). Biomarker values below LOD were included, as suggested by Olink (25). Initially, 44 patients and 44 controls were included, but one patient sample failed to pass the initial quality control by Olink and was therefore excluded from the study together with its respective control prior to any statistical analyses.

String

Any biological connections, i.e. similarities in functions or structure, for significant biomarkers were mapped by biomarker connection networks using the online database Search Tool for Retrieval of Interacting Genes/ Proteins (STRING; version 11.5) (26).

Statistics

Descriptive statistics are presented as percentage, mean (standard deviation, SD), median [interquartile range, IQR], or 95% confidence interval (95% CI). Normal distribution was evaluated using the Kolmogorov-Smirnov test. Group comparisons included patients with AAD and matched controls, overall and stratified for sex, and patients with and without RAF, and were conducted with Student's t-test, Mann–Whitney U-test, or Chi square test, as appropriate. Wilcoxon signed rank test and paired-samples T-test were used to investigate any significant change in biomarker values before and 60 minutes after the ACTH stimulation test. In Figure 1, P values are given as negative log transformed values.

For biomarkers significantly different between patients with AAD and matched controls, correlations between biomarker levels and number of adrenal crises the past year and AddiQoL-30 score were evaluated by Spearman's or Pearson's correlation coefficient, labelled r. Statistical significance was defined as $P < 0.05$, and multiple testing was corrected for by the Benjamini-Hochberg method using a false discovery rate (FDR) of 5% except for when comparing baseline characteristics and assessing correlations between biomarker levels and frequency of adrenal crises and AddiQoL-30 score.

Ethics

The study was approved by an ethical committee in each participating country prior to study start; Norway (permit no. 2018/751/REK Sør-Øst and REK 2016-00174), Sweden (permit no. 2018/2247-32), and Germany (permit no. Eth-47/18) and registered at clinicaltrials.gov (ClinicalTrials.gov no. NCT03793114 and NCT0218660). Written informed consent was obtained from all participants before inclusion.

Results

Baseline clinical characteristics of patients and healthy controls are presented in Table 1 and Supplementary Table 2. There were no significant differences in proportion of females, age, or BMI between patients and controls. Female patients and controls had significantly lower BMI compared with male patients and controls (23.1 kg/m² ± 2.3 vs. 25.1 kg/m² ± 3.1, P = 0.022 and 23.3 kg/m² ± 2.2 vs. 25.2 kg/m² ± 3.2, P = 0.031, respectively), but there were no significant differences in age (42 years ± 11 vs. 39 years ± 11, P = 0.462 and 41 years ± 11 vs. 40 years ± 11, P = 0.722, respectively).

Nineteen of the 177 biomarkers differed significantly between patients with AAD and controls at baseline, sorted by low-to-high P value: interleukin 6 (IL6), monocyte chemoattractant protein 1 (MCP1), receptor for advanced glycosylation end products (RAGE), adrenomedullin (ADM), galectin 9 (GAL9), tumor necrosis factor receptor superfamily member 9 (TNFRSF9), receptor activator of nuclear factor kappa-B (RANK), death receptor 4 (DR4), lymphotactin (XCL1), P-selectin glycoprotein ligand 1 (PSGL1), spondin 2 (SPON2), fibroblast growth factor 23 (FGF23), interleukin 12B (IL12B), matrix metallopeptidase 12 (MMP12), sulfotransferase 1A1 (ST1A1), fibroblast growth factor 21 (FGF21), death receptor 5 (DR5), RANK ligand (RANKL), and T-cell surface glycoprotein (CD4). Of these, all but ST1A1 were higher in patients compared with controls. The greatest difference in NPX values was noted for FGF21 (0.80 NPX, P

= 0.004) (Figure 1 and 2). Any biological connections between the 19 biomarkers are depicted in Supplementary Table 3 and Supplementary Figure.

Among the 19 biomarkers, number of adrenal crises the past year correlated with levels of RAGE (r = 0.415, P = 0.006), CD4 (r = 0.338, P = 0.029), and FGF21 (r = -0.317, P = 0.041). RAGE also negatively correlated with AddiQoL-30 scores (r = -0.347, P = 0.028) (correlations not corrected for multiple testing) (Table 3).

Stratifying for sex showed that female patients had significantly higher levels of 8 biomarkers (IL6, MCP1, GAL9, SPON2, DR4, placental growth factor (PGF), RAGE, and TNFRSF9) compared with female controls (Table 2), but no significant differences in biomarker levels were found between male patients and controls (data not shown).

Levels of leptin and growth hormone (GH) were significantly higher in female patients compared with male patients and in female controls compared with male controls (Leptin: 7.1 NPX ± 1.1 vs. 5.4 NPX ± 0.6, P < 0.0001 and 6.9 NPX ± 0.9 vs. 5.4 NPX ± 1.3, P < 0.0001, respectively. GH: 9.9 NPX ± 1.9 vs. 7.1 NPX ± 1.5, P < 0.0001 and 9.8 NPX ± 2.1 vs. 7.6 NPX ± 1.3, P=0.0002, respectively). No significant differences in biomarker levels were found between patients with and without RAF (Supplementary Table 4).

In AAD without RAF (n=19), there was a significant reduction in programmed death-ligand 2 (PDL2) (-0.15 NPX, *P* = 0.0001) and leptin (LEP) (-0.25 NPX, *P* = 0.0003) 60 minutes after injection of ACTH (Supplementary Table 4).

Discussion

We identified 19 cardiovascular and inflammatory biomarkers that differ between patients with AAD and healthy controls. All but one biomarker were elevated in patients, indicating an unfavorable cardiovascular milieu and pro-inflammatory state at the molecular level, with the greatest difference noted for FGF21. Alterations in biomarker profiles were sex-specific, with eight biomarkers differing between female patients and controls but none between male patients and controls. RAGE might be a marker of disease severity in AAD, with higher levels linked to more adrenal crises and reduced quality of life. We show that increasing ACTH levels from high to very high reduces PDL2 and leptin in a GC-independent manner, but the overall effect on biomarker profiles was small.

Metabolic regulator FGF21 was the single most elevated biomarker in AAD compared with controls. Beyond key roles in metabolic homeostasis (27), FGF21 has recently been implicated in the development and renewal of adrenocortical tissue (28). Even in the setting of adrenal insufficiency, injection of recombinant FGF21 is reported to stimulate cortisol secretion and lower ACTH in mice (29). Hence, elevated FGF21 could represent a compensatory attempt to keep up GC production as adrenocortical tissue is progressively lost. Worryingly, a recent meta-analysis found FGF21 to be an independent predictor of cardiometabolic disease progression and even death (30). On the other hand, FGF21 has been shown to have cardioprotective effects with promising therapeutic potential in cardiometabolic diseases (27). Taken together, studies on longitudinal associations between FGF21 levels and cardiometabolic outcome in patients with AAD are needed.

Elevated IL6 levels been found in primary adrenal insufficiency (PAI) by others as well (31, 32). The link between IL6 and CVD risk is well described, with mounting evidence from large-scale human studies even pointing to causality (33, 34). IL6 exerts a wide range of proinflammatory and immunoregulatory

actions and is proposed as therapeutic target in a multitude of diseases, including autoimmunity (35). In refractory rheumatoid arthritis, IL6 receptor inhibition may alleviate symptoms and reduce disease activity but at the cost of increased susceptibility to infections (35). This side-effect could be particularly detrimental in AAD and outweigh any potential benefit of IL6 inhibition, as infections are the number one cause of adrenal crises (35, 36).

Our finding of elevated MCP1 is in line with a recent report on 15 patients with AAD (37), but the clinical implications of this remains to be explored. Available literature suggest that MCP1 take part in the pathophysiology of several autoimmune diseases as well as CVD [30], with experimental models showing tapered inflammation in e.g. systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis, and reduced atherosclerotic plaque formation following inhibition of MCP1 or its receptor, CCR2 (38). Outcome in human trials, however, have been mixed and occasionally detrimental, fueling the debate as to whether MCP1 is mainly friend or foe (39).

Named for its role as receptor for advanced glycosylation end-product (AGEs), RAGE is best known as mediator of vascular inflammation and endothelial dysfunction, linked to development of atherosclerosis in general and diabetic complications in particular (40). We were intrigued to find the medium-strong correlations between RAGE levels and frequency of adrenal crises and AddiQoL-30 scores in AAD. Although not significant after correcting for multiple testing, we think it likely that the pro-inflammatory state negatively affect patient outcome in AAD, as suggested for a broad range of other conditions (41).

Other biomarkers that differed between patients with AAD and healthy controls included mediators of apoptosis (GAL9, DR4, DR5 (42, 43)), inflammatory agents (TNFRSF9, XCL1, CD4, IL12b (42, 44-46)), regulators of bone turnover (RANK, RANKL, FGF23 (47)), a hypotensive peptide (ADM (47)), and proteins

involved in general physiology, i.e. tissue remodeling (MMP12 (48)), cell adhesion (PSGL1, SPON2 (49, 50)), and sulfation (ST1A1 (51)).

We find sex-specific differences in biomarker profiles, corresponding to the recent finding that increased CVD risk in AAD is mainly carried by women (3). Indeed, seven biomarkers were higher in patients compared with controls, both for AAD overall and for the subgroup of women. In contrast, there were no significant difference in biomarker levels between male patients and male controls. As any clinical consequences of altered biomarker profiles are currently unknown, our findings call for more research on sex-specific differences in the risk and incidence of CVD in AAD.

Our finding of higher leptin and GH levels in women compared with men, in both patients and controls, correspond to established sex-differences in the concentrations of these hormones (52, 53). Of note, female patients and controls had significantly lower BMI than male patients and controls, and this may have influenced the observed difference in GH as levels are known to negatively correlate with BMI (53), but not leptin as levels positively correlate with BMI (52).

Biomarker levels did not differ between patients with and without RAF, possibly due to insufficient sample size. Why a subgroup of patients preserve some endogenous production of adrenocortical steroids even decades after diagnosis and any clinical implications of this remain unanswered.

Approved by the FDA in 1952, ACTH has historically been used to treat inflammatory diseases (e.g. rheumatoid arthritis, gout, psoriasis, ulcerative colitis) (54). For long, the anti-inflammatory effect was considered the mere result of GC-induction, until 2002 when Getting et al demonstrated preserved anti-inflammatory effects of ACTH after adrenalectomy (55). In the present study, change from high to very

high ACTH did not largely change cardiovascular and inflammatory biomarker profiles in in AAD without RAF, except for significant reductions in PDL2 and leptin levels.

To the best of our knowledge, this is the first study to suggest a regulatory role of ACTH on PDL2. As the better-known programmed death-ligand 1 (PDL1), PDL2 binds to programmed death protein 1 (PD1) and by this regulates immunity by putting the brake on T cell action. PDL1 variants have been linked to AAD risk, although not reproduced in the recent GWAS for AAD (56). We have not been able to find any publications successfully linking PDL2 to any autoimmune endocrinopathy.

Mainly secreted by adipocytes, leptin conveys information on energy stores to the brain and regulates appetite by acting on the melanocortin system and the HPA-axis. In turn, leptin secretion is stimulated by GC and inhibited by ACTH (57, 58). The latter was nicely depicted in the present study, as leptin significantly decreased following high to very high ACTH exposure in patients without RAF. There was a non-significant tendency towards higher leptin in AAD compared with controls at baseline, corresponding to a previous report on 63 patients with AAD (59). Taken together, we speculate that the observed decrease in leptin is an acute effect of ACTH that is lost in chronically elevated levels.

The present study included 12 CVD biomarkers that are previously reported to differ between patients with PAI and controls (32). Here, we were only able to replicate elevated IL6. Two other markers (ADM and MMP12) significantly differed as well but in the opposite direction. We suspect the contrasting findings to be due to differences in GC exposure (60, 61), as blood samples in the previous study were collected shortly after patients had taken their morning GC replacement (32) whereas patients here had abstained from any GC replacement for at least 24 hours prior to sampling. In addition, the previous study included participants with metabolic syndrome and diabetes mellitus (type 1 and 2), significantly

overrepresented in patients compared with controls, that could potentially have affected the results (32).

Strengths of the present study include a well-characterized patient cohort of autoimmune AD etiology only, without concomitant DM, metabolic syndrome, or overt CVD, and comparing biomarker profiles with matched controls. Of note, one patient had a history of statin use, but excluding this patient did not alter the results. Taking the recently recognized phenomenon of RAF into account allowed us to study GC-independent effects of ACTH, although at highly elevated levels and the synthetic form, on cardiovascular and inflammatory biomarkers *in vivo*.

The study has several limitations that merit consideration. For one, the sample sizes of 43 patients and 43 matched controls are relatively small. Second, the cross-sectional study design does not allow prediction of future cardiovascular events or exploration of temporal dynamics in the autoimmune process. Interpretation of data is further complicated by the lack of biomarker reference ranges, which we have partly compensated for by including matched controls. Importantly, matching can never be done perfectly, here exemplified by oral contraceptive pills used by more female patients (n=6) than female controls (n=2). Although we standardized for time of sampling (morning) and food intake, we acknowledge that the results could be affected by several unknown pre-analytical differences as well, including smoking and family history of CVD. We further acknowledge that a true regulatory effect of ACTH on biomarkers may have gone undetected as patients already had high ACTH prior to the ACTH stimulation testing. As the biomarker half-lives are largely unknown, it is also possible that 60 minutes was too short to detect all true changes following injection of ACTH. Implemented as an extra safety measure for patients upon GC withdrawal, we cannot rule out an interfering role of food intake as several of the analyzed markers are reported to change following eating, including a reduction in leptin

(62). Finally, several biomarkers were nearly significant when correcting for multiple testing, and it is possible that interesting connections may have been erroneously overlooked.

To conclude, patients with AAD and especially women have increased levels of cardiovascular and inflammatory biomarkers profiles compared with controls, with the greatest difference found for FGF21 levels. RAGE might be a marker of disease severity in AAD, associated with frequency of adrenal crises and reduced quality of life. Very high ACTH exposure seems to reduce PDL2 and leptin in a GC-independent manner, but the overall impact of elevated ACTH on cardiovascular health and inflammation in AAD remains to be determined.

Additional information

Disclosure statement: The authors have nothing to disclose.

Data availability: The data sets on baseline characteristics and biomarker values are not publicly available but fully anonymized versions may be shared upon reasonable request.

Acknowledgements

We thank Lars Breivik, Elisabeth Tombra Halvorsen, and Marie Karlsen (Endocrinological Research Laboratory, Department of Clinical Science, University of Bergen) for helping to organize the study, handling blood samples, and preparing shipment of samples. We thank Professor Rolv Terje Lie and bioinformatician Julia Romanowska at the Department of Global Public Health and Primary Care (University of Bergen) for statistical advice, and the Olink staff (Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala, Sweden) and Olink Analysis Service in particular for their skillful technical support. We are grateful to Mona Eliassen, Nina Jensen, Gro M. Olderøy, Inger Svendsen (Haukeland University Hospital), Lillian Skumsnes (Haugesund Hospital), Hanne Høivik Bjørkås and Elise Turkerud

Søby (Innlandet Hospital Trust), Maria Wärn (Karolinska University Hospital), Christina Dahlgren (University Hospital Linköping), Katarina Iselid (Umeå University Hospital), Anette Nilsson (Central Hospital Karlstad), Kari Irene Abelsen (Oslo University Hospital), Anne Breikert (University Hospital Örebro), and Britta Bauer (Endocrinology in Charlottenburg, Berlin, Germany) for good patient care and collection of blood samples. We thank Åsa Hallgren (Karolinska Institutet) and Øyvind Skadberg (Stavanger University Hospital) for logistics support. We also thank Nina Henne and Nebeyaet Selemon Gebreslase (Core Facility for Metabolomics, Department of Clinical Science, University of Bergen) for analyzing samples on LC-MS/MS, and to Anders Engeland (Department of Global Public Health and Primary Care, University of Bergen) for statistical counseling. Last, but not least, we thank all the participants who made this study possible.

Financial support

Research Council of Norway (grant no. 288022, ESH), The Novo Nordisk Foundation (grant no. NNF18OC0034130, EHS), The Internal Medicine Association of Norway (ESH), The Legate of Dr. Nils Henrichsen and Wife Anna Henrichsen (ÅBS), University of Bergen (ÅBS, GU), Western Regional Health Authorities (GU, MØ, ESH), and Department of Medicine and Hormone Laboratory, Haukeland University Hospital (GU, MØ, ESH), and the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet (SB, AKÅ).

References

1. Husebye ES, Pearce SH, Krone NP, Kämpe O. Adrenal insufficiency. Lancet (London, England). 2021;397(10274):613-29.

2. Bjornsdottir S, Sundstrom A, Ludvigsson JF, Blomqvist P, Kampe O, Bensing S. Drug prescription patterns in patients with Addison's disease: a Swedish population-based cohort study. J Clin Endocrinol Metab. 2013;98(5):2009-18.

3. Skov J, Sundstrom A, Ludvigsson JF, Kampe O, Bensing S. Sex-specific risk of cardiovascular disease in autoimmune Addison's disease - a population-based cohort study. The Journal of clinical endocrinology and metabolism. 2019.

4. Dalin F, Nordling Eriksson G, Dahlqvist P, Hallgren A, Wahlberg J, Ekwall O, et al. Clinical and immunological characteristics of Autoimmune Addison's disease: a nationwide Swedish multicenter study. The Journal of clinical endocrinology and metabolism. 2016:jc20162522.

5. Rahvar AH, Haas CS, Danneberg S, Harbeck B. Increased Cardiovascular Risk in Patients with Adrenal Insufficiency: A Short Review. BioMed research international. 2017;2017:3691913.

6. Choudhury S, Lightman S, Meeran K. Improving glucocorticoid replacement profiles in adrenal insufficiency. Clinical endocrinology. 2019.

7. Ngaosuwan K, Johnston DG, Godsland IF, Cox J, Majeed A, Quint JK, et al. Cardiovascular Disease in Patients With Primary and Secondary Adrenal Insufficiency and the Role of Comorbidities. The Journal of clinical endocrinology and metabolism. 2021;106(5):1284-93.

8. Conrad N, Verbeke G, Molenberghs G, Goetschalckx L, Callender T, Cambridge G, et al. Autoimmune diseases and cardiovascular risk: a population-based study on 19 autoimmune diseases and 12 cardiovascular diseases in 22 million individuals in the UK. Lancet (London, England). 2022;400(10354):733-43.

9. Molnár Á, Kövesdi A, Szücs N, Tóth M, Igaz P, Rácz K, et al. Polymorphisms of the GR and HSD11B1 genes influence body mass index and weight gain during hormone replacement treatment in patients with Addison's disease. Clinical endocrinology. 2016;85(2):180-8.

10. Fichna M, Żurawek M, Gryczyńska M, Sowińska A, Nowak J, Ruchała M. Polymorphic variants of the HSD11B1 gene may be involved in adverse metabolic effects of glucocorticoid replacement therapy in Addison's disease. Eur J Intern Med. 2016;31:99-104.

11. Sævik Å B, Åkerman AK, Methlie P, Quinkler M, Jørgensen AP, Höybye C, et al. Residual Corticosteroid Production in Autoimmune Addison Disease. The Journal of clinical endocrinology and metabolism. 2020;105(7).

12. Bancos I, Hazeldine J, Chortis V, Hampson P, Taylor AE, Lord JM, et al. Primary adrenal insufficiency is associated with impaired natural killer cell function: a potential link to increased mortality. European journal of endocrinology / European Federation of Endocrine Societies. 2017;176(4):471-80.

13. Edvardsen K, Bjånesøy T, Hellesen A, Breivik L, Bakke M, Husebye ES, et al. Peripheral Blood Cells from Patients with Autoimmune Addison's Disease Poorly Respond to Interferons In Vitro, Despite Elevated Serum Levels of Interferon-Inducible Chemokines. J Interferon Cytokine Res. 2015;35(10):759-70.

14. Bellastella G, Rotondi M, Pane E, Costantini S, Colella C, Calemma R, et al. Simultaneous evaluation of the circulating levels of both Th1 and Th2 chemokines in patients with autoimmune Addison's disease. Journal of endocrinological investigation. 2011;34(11):831-4.

15. Ekman B, Alstrand N, Bachrach-Lindström M, Jenmalm MC, Wahlberg J. Altered chemokine Th1/Th2 balance in Addison's disease: relationship with hydrocortisone dosing and quality of life. Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme. 2014;46(1):48-53.

16. Rotondi M, Falorni A, De Bellis A, Laureti S, Ferruzzi P, Romagnani P, et al. Elevated serum interferon-gamma-inducible chemokine-10/CXC chemokine ligand-10 in autoimmune primary adrenal insufficiency and in vitro expression in human adrenal cells primary cultures after stimulation with proinflammatory cytokines. The Journal of clinical endocrinology and metabolism. 2005;90(4):2357-63.

17. Isidori AM, Venneri MA, Graziadio C, Simeoli C, Fiore D, Hasenmajer V, et al. Effect of once-daily, modified-release hydrocortisone versus standard glucocorticoid therapy on metabolism and innate immunity in patients with adrenal insufficiency (DREAM): a single-blind, randomised controlled trial. The lancet Diabetes & endocrinology. 2018;6(3):173-85.

18. Betterle C, Presotto F, Furmaniak J. Epidemiology, pathogenesis, and diagnosis of Addison's disease in adults. Journal of endocrinological investigation. 2019;42(12):1407-33.

19. Hasenmajer V, Bonaventura I, Minnetti M, Sada V, Sbardella E, Isidori AM. Non-Canonical Effects of ACTH: Insights Into Adrenal Insufficiency. Front Endocrinol (Lausanne). 2021;12:701263.

20. Ueland GA, Methlie P, Oksnes M, Thordarson HB, Sagen J, Kellmann R, et al. The Short Cosyntropin Test Revisited: New Normal Reference Range Using LC-MS/MS. The Journal of clinical endocrinology and metabolism. 2018;103(4):1696-703.

21. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. Nucleic Acids Res. 2011;39(15):e102.

22. Assarsson E, Lundberg M, Holmquist G, Björkesten J, Thorsen SB, Ekman D, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PloS one. 2014;9(4):e95192.

23. Olink. Olink validation document for the Inflammation panel (article no. 95302) 2019 [Available from: https://www.olink.com/content/uploads/2019/04/Olink-Inflammation-Validation-Data-v3.0.pdf.

443 24. Olink. Olink validation document for the CVD II panel (article no. 95500) 2019 [Available from:
444 https://www.olink.com/content/uploads/2019/12/Olink-CVD-II-Validation-Data-v2.1.pdf.
445 25. Olink. How is the Limit of Detection (LOD) estimated and how is this handled in the data
446 analysis? 2021 [Available from: https://www.olink.com/question/how-is-the-limit-of-detection-lod-
447 estimated-and-handled/.
448 26. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-
449 protein association networks with increased coverage, supporting functional discovery in genome-wide
450 experimental datasets. Nucleic Acids Res. 2019;47(D1):D607-d13.
451 27. Geng L, Lam KSL, Xu A. The therapeutic potential of FGF21 in metabolic diseases: from bench to
452 clinic. Nature reviews Endocrinology. 2020;16(11):654-67.
453 28. Pihlajoki M, Dörner J, Cochran RS, Heikinheimo M, Wilson DB. Adrenocortical zonation, renewal,
454 and remodeling. Front Endocrinol (Lausanne). 2015;6:27.
455 29. Díaz-catalán D V-BA, Mora M., Rodrigo M, Boswell L, Casals G, and Hanzu F. A. Effects of
456 Fibroblast factor 21 to adrenal renewal after chronic hypercortisolism. Endocrine Abstracts (2022) 83
457 AO4 2022.
458 30. Lakhani I, Gong M, Wong WT, Bazoukis G, Lampropoulos K, Wong SH, et al. Fibroblast growth
459 factor 21 in cardio-metabolic disorders: a systematic review and meta-analysis. Metabolism. 2018;83:11-
460 7.
461 31. Rahvar AH, Riesel M, Graf T, Harbeck B. Adrenal insufficiency treated with conventional
462 hydrocortisone leads to elevated levels of Interleukin-6: a pilot study. Endocrine. 2019;64(3):727-9.
463 32. Bergthorsdottir R, Ragnarsson O, Skrtic S, Glad CAM, Nilsson S, Ross IL, et al. Visceral Fat and
464 Novel Biomarkers of Cardiovascular Disease in Patients With Addison's Disease: A Case-Control Study.
465 The Journal of clinical endocrinology and metabolism. 2017;102(11):4264-72.

33. Sarwar N, Butterworth AS, Freitag DF, Gregson J, Willeit P, Gorman DN, et al. Interleukin-6 receptor pathways in coronary heart disease: a collaborative meta-analysis of 82 studies. Lancet (London, England). 2012;379(9822):1205-13.

34. Bacchiega BC, Bacchiega AB, Usnayo MJ, Bedirian R, Singh G, Pinheiro GD. Interleukin 6 Inhibition and Coronary Artery Disease in a High-Risk Population: A Prospective Community-Based Clinical Study. J Am Heart Assoc. 2017;6(3).

35. Choy EH, De Benedetti F, Takeuchi T, Hashizume M, John MR, Kishimoto T. Translating IL-6 biology into effective treatments. Nat Rev Rheumatol. 2020;16(6):335-45.

36. Quinkler M, Murray RD, Zhang P, Marelli C, Petermann R, Isidori AM, et al. Characterization of patients with adrenal insufficiency and frequent adrenal crises. European journal of endocrinology / European Federation of Endocrine Societies. 2021;184(6):761-71.

37. Kraus AU, Penna-Martinez M, Shoghi F, Meyer G, Badenhoop K. Monocytic Cytokines in Autoimmune Polyglandular Syndrome Type 2 Are Modulated by Vitamin D and HLA-DQ. Front Immunol. 2020;11:583709.

38. Bianconi V, Sahebkar A, Atkin SL, Pirro M. The regulation and importance of monocyte chemoattractant protein-1. Curr Opin Hematol. 2018;25(1):44-51.

39. Panee J. Monocyte Chemoattractant Protein 1 (MCP-1) in obesity and diabetes. Cytokine. 2012;60(1):1-12.

40. Dong H, Zhang Y, Huang Y, Deng H. Pathophysiology of RAGE in inflammatory diseases. Front Immunol. 2022;13:931473.

41. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nature reviews Neuroscience. 2008;9(1):46-56.

42. Sonar S, Lal G. Role of Tumor Necrosis Factor Superfamily in Neuroinflammation and Autoimmunity. Front Immunol. 2015;6:364.

43. Kashio Y, Nakamura K, Abedin MJ, Seki M, Nishi N, Yoshida N, et al. Galectin-9 induces apoptosis through the calcium-calpain-caspase-1 pathway. J Immunol. 2003;170(7):3631-6.

44. Lei Y, Takahama Y. XCL1 and XCR1 in the immune system. Microbes Infect. 2012;14(3):262-7.

45. Wehler TC, Karg M, Distler E, Konur A, Nonn M, Meyer RG, et al. Rapid identification and sorting of viable virus-reactive CD4(+) and CD8(+) T cells based on antigen-triggered CD137 expression. J Immunol Methods. 2008;339(1):23-37.

46. van Wanrooij RL, Zwiers A, Kraal G, Bouma G. Genetic variations in interleukin-12 related genes in immune-mediated diseases. J Autoimmun. 2012;39(4):359-68.

47. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun. 1993;192(2):553-60.

48. Lee J-T, Pamir N, Liu N-C, Kirk EA, Averill MM, Becker L, et al. Macrophage Metalloelastase (MMP12) Regulates Adipose Tissue Expansion, Insulin Sensitivity, and Expression of Inducible Nitric Oxide Synthase. Endocrinology. 2014;155(9):3409-20.

49. Tinoco R, Otero DC, Takahashi AA, Bradley LM. PSGL-1: A New Player in the Immune Checkpoint Landscape. Trends Immunol. 2017;38(5):323-35.

50. Consortium TU. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Research. 2022:spondin 2 homo sapiens.

51. Mueller JW, Gilligan LC, Idkowiak J, Arlt W, Foster PA. The Regulation of Steroid Action by Sulfation and Desulfation. Endocrine reviews. 2015;36(5):526-63.

52. Hellström L, Wahrenberg H, Hruska K, Reynisdottir S, Arner P. Mechanisms behind gender differences in circulating leptin levels. Journal of internal medicine. 2000;247(4):457-62.

513 53. Bänsch D, Dirkes-Kersting A, Schulte H, Assmann G, von Eckardstein A. Basal growth hormone
514 levels are positively correlated with high-density lipoprotein cholesterol levels in women. Metabolism.
515 1997;46(9):1039-43.
516 54. Montero-Melendez T. ACTH: The forgotten therapy. Semin Immunol. 2015;27(3):216-26.
517 55. Getting SJ, Christian HC, Flower RJ, Perretti M. Activation of melanocortin type 3 receptor as a
518 molecular mechanism for adrenocorticotropic hormone efficacy in gouty arthritis. Arthritis Rheum.
519 2002;46(10):2765-75.
520 56. Eriksson D, Røyrvik EC, Aranda-Guillén M, Berger AH, Landegren N, Artaza H, et al. GWAS for
521 autoimmune Addison's disease identifies multiple risk loci and highlights AIRE in disease susceptibility.
522 Nat Commun. 2021;12(1):959.
523 57. Angelousi A, Margioris AN, Tsatsanis C. ACTH Action on the Adrenals. In: Feingold KR, Anawalt B,
524 Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., editors. Endotext. South Dartmouth (MA):
525 MDText.com, Inc.
526 Copyright © 2000-2022, MDText.com, Inc.; 2000.
527 58. Uddén J, Björntorp P, Arner P, Barkeling B, Meurling L, Rössner S. Effects of glucocorticoids on
528 leptin levels and eating behaviour in women. Journal of internal medicine. 2003;253(2):225-31.
529 59. Fichna M, Fichna P, Gryczyńska M, Czarnywojtek A, Żurawek M, Ruchała M. Steroid replacement
530 in primary adrenal failure does not appear to affect circulating adipokines. Endocrine. 2015;48(2):677-85.
531 60. Letizia C, Cerci S, Centanni M, De Toma G, Subioli S, Scuro L, et al. Circulating levels of
532 adrenomedullin in patients with Addison's disease before and after corticosteroid treatment. Clinical
533 endocrinology. 1998;48(2):145-8.
534 61. Uhlenhaut NH, Barish GD, Yu RT, Downes M, Karunasiri M, Liddle C, et al. Insights into negative
535 regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. Mol
536 Cell. 2013;49(1):158-71.

537 62. Dencker M, Björgell O, Hlebowicz J. Effect of food intake on 92 neurological biomarkers in
538 plasma. Brain Behav. 2017;7(9):e00747.

539

Tables

Table 1. Baseline characteristics for study participants

Characteristics	Patients with AAD (n=43)	Healthy controls (n=43)	*P* value
Female sex, no. (%)	19 (44)	19 (44)	1.0
Age, years	40 ± 23	40 ± 11	0.8
BMI, kg/m²	24.2 ± 2.9	24.3 ± 2.9	0.8

Abbreviations: AAD; autoimmune Addison's disease, BMI; body mass index, no; number.

Data are given as number (percentage %) and mean ±SD.

546 Table 2. Biomarkers significantly different between female patients with AAD and female

547 healthy controls

548

Biomarker	Female AAD (n=19)	Female HC (n=19)	*P value*
IL6	3.8 [3.6-4.6]	3.2 [2.6-3.4]	<0.0001
MCP1	13.3 ± 0.4	12.6 ± 0.6	<0.0001
GAL9	8.3 ± 0.3	7.9 ± 0.3	0.0002
SPON2	9.0 ± 0.1	8.9 ± 0.2	0.0005
DR4	3.3 ± 0.4	2.9 ± 0.3	0.0008
PGF	8.0 ± 0.4	7.6 ± 0.3	0.001
RAGE	14.1 ± 0.4	13.5 ± 0.3	0.001
TNFRSF9	7.4 ± 0.5	7.2 ± 0.4	0.002

549 Abbreviations: AAD; autoimmune Addison's disease, DR4; death receptor 4, GAL9; galectin 9, HC; healthy

550 controls, IL6; interleukin 6 (IL6), MCP1; monocyte chemoattractant protein 1, PGF; placental growth

551 factor, RAGE; receptor for advanced glycosylation end products, SPON2; spondin 2, TNFRSF9; tumor

552 necrosis factor receptor superfamily member 9.

553 Data are given as mean ±SD or median [IQR].

554 Table 3. Correlations between patient characteristics and levels of 19 biomarkers different in AAD

		ADM	CD4	FGF21	FGF23	GAL9	IL12B	IL6	MCP1	MMP12	PSGL1	RAGE	RANKL	SPON2	ST1A1	TNFRSF9	DR4	DR5	RANK	XCL1
No. AC	*r*	0.012	0.338*	-0.317*	-0.019	0.294	0.244	0.015	-0.06	0.137	0.257	0.415*	0.082	0.012	-0.006	0.186	0.047	0.116	0.012	0.199
past year	P	0.938	0.029	0.041	0.902	0.059	0.115	0.923	0.702	0.386	0.101	0.006	0.603	0.941	0.972	0.232	0.766	0.465	0.939	0.206
	N	42	42	42	43	42	43	42	43	42	42	42	43	42	43	43	42	42	42	42
AddiQol-	*r*	-0.195	-0.159	-0.041	-0.274	0.030	-0.199	-0.051	-0.211	0.061	-0.090	-0.347*	-0.192	-0.186	-0.232	-0.187	-0.132	0.079	-0.194	0.102
30	P	0.227	0.328	0.801	0.083	0.855	0.212	0.755	0.185	0.708	0.581	0.028	0.228	0.25	0.144	0.242	0.416	0.629	0.229	0.53
	N	40	40	40	41	40	41	40	41	40	40	40	41	40	41	41	40	40	40	40

555

556 Abbreviations: AC; adrenal crises, ADM; adrenomedullin, CD4; T-cell surface glycoprotein, FGF21; fibroblast growth factor 21, FGF23; fibroblast
557 growth factor 23 (FGF23), GAL9; galectin 9 (GAL9), IL12B; interleukin 12B, IL6; interleukin 6, MCP1; monocyte chemoattractant protein 1,
558 MMP12; matrix metallopeptidase 12, PSGL1; P-selectin glycoprotein ligand 1, RAGE; receptor for advanced glycosylation end products, RANKL;
559 receptor activator of nuclear factor kappa-B ligand, SPON2; spondin 2, ST1A1; sulfotransferase 1A1, TNFRSF9; tumor necrosis factor receptor
560 superfamily member 9, DR4; tumor necrosis factor receptor superfamily member 10A, DR5; tumor necrosis factor receptor superfamily member
561 10B, RANK; tumor necrosis factor receptor superfamily member 11A, XCL1; lymphotactin.
562 *$P < 0.050$.

563

564

565

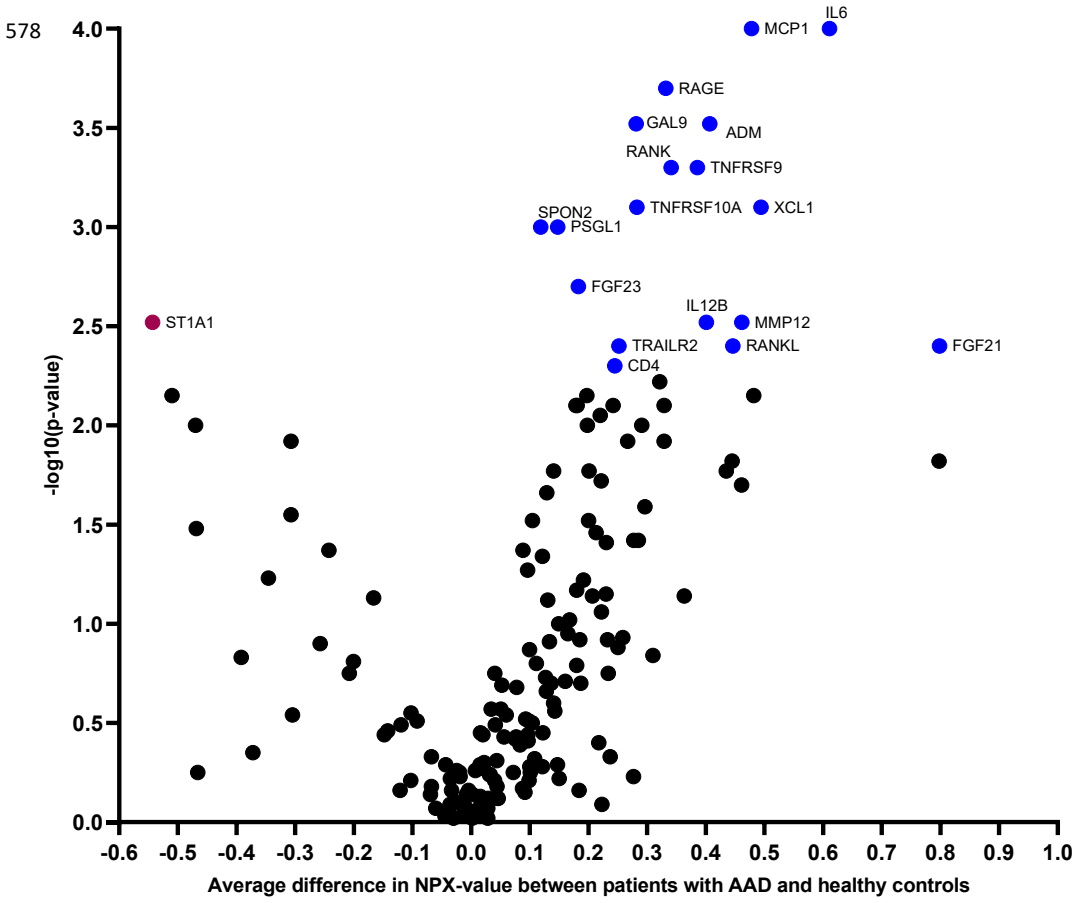
Legends

Figure 1. Volcano plot depicting differences in average NPX values between patients with AAD and healthy controls for the 177 analyzed biomarkers, of which 18 were significantly higher (blue dots) and one (red dot) significantly lower in patients compared with control. Black dots represent biomarkers that were not significantly different between patients and controls.

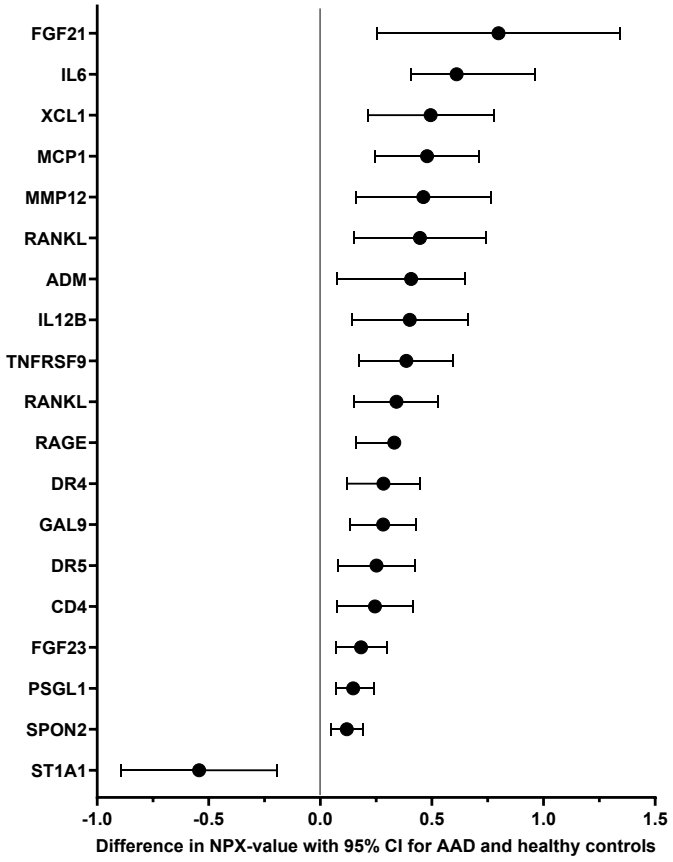
Figure 2. Forest plot depicting differences in average NPX values with 95% confidence interval (95% CI) between 19 biomarkers significantly different between patients with AAD and healthy controls.

Figure

Figure 1



579 Figure 2



580

Supplementary

Tables

Supplementary Table 1

All 177 analyzed biomarkers with limits of detection (LOD) and coefficients of variances (CV) within a plate of samples (intra) and between plates (inter).

Data for the CARDIOVASCULR DISEASE II (CVD II) panel is obtained from https://olink.com/content/uploads/2021/09/olink-cvd-ii-validation-data-v2.1.pdf

Biomarker name (abbreviation)	**Biomarker synonym**	**LOD in pg/mL**	**CV % intra**	**CV % inter**
2,4-dienoyl-CoA reductase, mitochondrial (DECR1)	Short chain dehydrogenase/reductase family 18C member 1	488.28	15	15
A disintegrin and metalloproteinase with thrombospondin motifs 13 (ADAM-TS13)	von Willebrand factor-cleaving protease	122.07	4	10
Adrenomedullin (ADM)		488.28	13	11
Agouti-related protein (AGRP)		488.28	9	12
Alpha-L-iduronidase (IDUA)		0.95	6	18
Angiopoietin-1 (ANGPT1)		61.04	9	9

Angiopoietin-1 receptor (TIE2)	Tyrosine-protein kinase receptor TEK	7.63	8	14
Angiotensin-converting enzyme 2 (ACE2)	ACE-related carboxypeptidase	15.26	8	11
Bone morphogenetic protein 6 (BMP-6)	VG-1-related protein	1.91	21	15
Brother of CDO (BOC		61.04	10	14
Carbonic anhydrase 5A (CA5A)	Carbonic anhydrase VA (CAVA)	0.95	9	10
Carcinoembryonic antigenrelated cell adhesion molecule 8 (CEACAM8)	CD67 antigen	244.14	11	10
Cathepsin L1 (CTSL1)		244.14	10	10
C-C motif chemokine 17 (CCL17)	Thymus and activation-regulated chemokine	1.91	12	13
C-C motif chemokine 3 (CCL3)	Macrophage inflammatory protein 1-alpha	0.48	9	8
CD40 ligand (CD40-L)	Tumor necrosis factor ligand superfamily member 5 (TNFSF5)	0.48	9	14
Chymotrypsin C (CTRC)	Caldecrin	3.81	10	10

C-X-C motif chemokine 1 (CXCL1)	Growth-regulated alpha protein	7.63	10	13
Death receptor 4	Tumor necrosis factor receptor superfamily member 10A (TNFRSF10A), TNF-related apoptosis-inducing ligand receptor 2 (TRAILR1)	0.95	11	11
Death receptor 5	Tumor necrosis factor receptor superfamily member 10B (TNFRSF10B), TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2)	0.24	10	12
Decorin (DCN)	Bone proteoglycan II	122.07	7	11
Dickkopf-related protein 1 (Dkk-1)		15.26	11	9
Fatty acid-binding protein, intestinal (FABP2)		0.24	8	9
Fibroblast growth factor 21 (FGF21)		122.07	12	14
Fibroblast growth factor 23 (FGF23)	Phosphatonin	122.07	14	15
Follistatin (FS)	Activin-binding protein	5 0.95	9	15

Galectin-9 (Gal9)	Ecalectin	15.26	5	13
Gastric intrinsic factor (GIF)	Cobalamin binding intrinsic factor (CBLIF)	0.48	11	15
Gastrotropin (GT)	Fatty acid-binding protein 6 (FABP6)	488.28	16	15
Growth hormone (GH)	Somatotropin	1.91	7	9
Growth/differentiation factor 2 (GDF-2)	Bone morphogenetic protein 9	7.63	9	11
Heat shock 27 kDa protein (HSP27)	Heat shock protein beta-1 (HSPB1)	122.07	11	12
Heme oxygenase 1 (HO-1)	HMOX1	1.91	8	10
Hydroxyacid oxidase 1 (HAOX1)	HAO1	122.07	9	9
Integrin beta-1-binding protein 2 (ITGB1BP2)	Melusin	976.56	11	11
Interleukin-1 receptor antagonist protein (IL1ra)	IL1 inhibitor	3.81	12	36
Interleukin-1 receptor-like 2 (IL1RL2)	IL-36 receptor	122.07	10	14
Interleukin-17D (IL17D)		3.81	13	12
Interleukin-18 (IL18)		0.48	11	11
Interleukin-27 (IL27)		122.07	7	11

Interleukin-4 receptor subunit alpha (IL4RA)	IL-4-binding protein	7.63	9	15
Interleukin-6 (IL6)		0.24	9	9
Kidney injury molecule 1 (KIM1)	Hepatitis A virus cellular receptor 1 (HAVCR1)	0.48	11	9
Lactoylglutathione lyase (GLO1)		0.95	8	11
Lectin-like oxidized LDL receptor 1 (LOX1)	Oxidized low-density lipoprotein receptor 1	0.95	9	11
Leptin (LEP)		61.04	6	10
Lipoprotein lipase (LPL)		0.48	7	8
Low affinity immunoglobulin gamma Fc region receptor II-b (IgG Fc receptor IIb)	FCGR2B	15.26	9	15
Lymphotactin (XCL1)	C motif chemokine 1	61.04	10	10
Macrophage receptor MARCO (MARCO)		30.52	6	5
Matrix metalloproteinase-12 (MMP12)		15.26	11	10
Matrix metalloproteinase-7 (MMP7)		7.63	9	9

Natriuretic peptides B (BNP)		488.28	N/A	N/A
NF-kappa-B essential modulator (NEMO)		3.00	9	9
Osteoclast-associated immunoglobulinlike receptor (hOSCAR)	OSCAR	1.91	5	10
Pappalysin-1 (PAPPA)	Insulin-like growth factor-dependent IGF-binding protein 4 protease	61.04	13	12
Pentraxin-related protein PTX3 (PTX3)	Tumor necrosis factor alpha-induced protein 5	1.91	8	10
Placenta growth factor (PGF)		0.48	12	13
Platelet-derived growth factor subunit B (PDGF subunit B)		15.26	11	12
Poly [ADP-ribose] polymerase 1 (PARP-1)		61.04	9	11
Polymeric immunoglobulin receptor (PIgR)	Hepatocellular carcinoma-associated protein TB6	122.07	3	14
Programmed cell death 1 ligand 2 (PDL2)		488.28	9	10

Proheparin-binding EGF-like growth factor (HBEGF)		0.48	8	10
Pro-interleukin-16 (IL16)		3.81	11	12
Prolargin (PRELP)		15.26	7	8
Prostasin (PRSS8)	Serine protease 8	0.24	8	11
Protein AMBP (AMBP)		1953.12	6	7
Proteinase-activated receptor 1 (PAR1)	Thrombin receptor	30.52	9	12
Protein-glutamine gammaglutamyltransferase 2 (TGM2)		61.04	8	12
Proto-oncogene tyrosine-protein kinase Src (SRC)		15.26	10	12
P-selectin glycoprotein ligand 1 (PSGL1)	Selectin P ligand	1.91	6	10
Receptor for advanced glycosylation end products (RAGE)	Advanced glycosylation end products receptor (AGER)	0.48	9	11
Renin (REN)		7.63	8	12
Serine protease 27 (PRSS27)		1.91	9	13
Serine/threonine-protein kinase 4 (STK4)		488.28	7	10

Serpin A12 (SERPINA12)		15.26	10	22
SLAM family member 5 (CD84)	Leukocyte differentiation antigen CD84	61.04	9	12
SLAM family member 7 (SLAMF7)		244.14	11	9
Sortilin (SORT1)		122.07	8	12
Spondin-2 (SPON2)		30.52	5	11
Stem cell factor (SCF)	Kit ligand (KITLG)	1.91	7	12
Superoxide dismutase [Mn], mitochondrial (SOD2)		15.26	6	9
T-cell surface glycoprotein CD4 (CD4)		15.26	10	9
Thrombomodulin (TM)	Fetomodulin	7.63	11	10
Thrombopoietin (THPO)		122.07	9	11
Thrombospondin-2 (THBS2)		61.04	5	8
Tissue factor (TF)	Coagulation factor III	0.24	8	13
Receptor activator of nuclear factor κ B (RANK)	Tumor necrosis factor receptor superfamily member 11A (TNFRSF11A), RANKL receptor	0.95	10	13

Tumor necrosis factor receptor superfamily member 13B (TNFRSF13B)		61.04	10	10
Tyrosine-protein kinase Mer (MERTK)		244.14	10	10
Vascular endothelial growth factor D (VEGFD)		61.04	7	10
V-set and immunoglobulin domaincontaining protein 2 (VSIG2)		30.52	8	10

588 Abbreviations: N/A; not available

589

590 Data from the Inflammation biomarker panel is obtained from

591 https://olink.com/content/uploads/2021/09/olink-inflammation-validation-data-v3.0.pdf

592

Biomarker	Synonyms	LOD	CV % intra	CV % inter
Adenosine Deaminase (ADA)		0.48	5	29
Artemin (ARTN)		0.24	7	18
Axin-1 (AXIN1)		61	6	19
Beta-nerve growth factor (Beta-NGF)		0.48	6	14

Caspase 8 (CASP8)	Apoptotic cysteine protease	0.48	7	22
C-C motif chemokine 4 (CCL4)		1.9	6	17
C-C motif chemokine 19 (CCL19)		15	8	15
C-C motif chemokine 20 (CCL20)		7.6	7	13
C-C motif chemokine 23 (CCL23)		31	6	13
C-C motif chemokine 25 (CCL25)		3.8	6	18
C-C motif chemokine 28 (CCL28)		61	7	14
CD40L receptor (CD40)	Tumor necrosis factor receptor superfamily member 5 (TNFRSF5)	0.01	5	21
CUB domain-containing protein 1 (CDCP1)		0.12	6	24
C-X-C motif chemokine 1 (CXCL1)	Growth-regulated alpha protein	3.8	6	15

C-X-C motif chemokine 5 (CXCL5)		0.95	7	13
C-X-C motif chemokine 6 (CXCL6)		7.6	8	14
C-X-C motif chemokine 9 (CXCL9)		0.95	6	12
C-X-C motif chemokine 10 (CXCL10)		7.6	7	11
C-X-C motif chemokine 11 (CXCL11)		7.6	7	14
Cystatin D (CST5)	Cystatin-5	1.9	5	21
Delta and Notch-like epidermal growth factorrelated recep (DNER)		0.95	5	26
Eotaxin-1 (CCL11)		3.8	5	14
Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)		1.79	6	23
Fibroblast growth factor 5 (FGF5)		1.9	7	14
Fibroblast growth factor 19 (FGF19)		7.6	6	19

Fibroblast growth factor 21 (FGF21)		31	8	21
Fibroblast growth factor 23 (FGF23)		122	9	26
Fms-related tyrosine kinase 3 ligand (Flt3L)	SL cytokine	0.01	6	15
Fractalkine (CX3CL1)	C-X3-C motif chemokine 1	15.3	7	24
Glial cell line-derived neurotrophic factor (GDNF)		0.01	7	12
Hepatocyte growth factor (HGF)		7.6	6	16
Interferon gamma (IFN-gamma)		0.24	7	24
Interleukin-1 alpha (IL1 alpha)		0.48	7	18
Interleukin-2 (IL2)		30.5	9	16
Interleukin-2 receptor subunit beta (IL2RB)		15	7	19
Interleukin-4 (IL4)		0.24	7	16
Interleukin-5 (IL5)		3.8	7	17
Interleukin-6 (IL6)		0.12	6	8

Interleukin-7 (IL7)		0.24	6	18
Interleukin-8 (IL8)		0.03	6	15
Interleukin-10 (IL10)		0.48	7	12
Interleukin-10 receptor subunit alpha (IL10RA)		3.8	6	19
Interleukin-10 receptor subunit beta (IL10RB)		0.12	5	31
Interleukin-12 subunit beta (IL12B)		0.12	6	16
Interleukin-13 (IL13)		7.6	14	26
Interleukin-15 receptor subunit alpha (IL15RA)		0.95	6	20
Interleukin-17A (IL17A)		3.8	8	17
Interleukin-17C (IL17C)		31	8	18
Interleukin-18 (IL18)		0.06	6	19
Interleukin-18 receptor 1 (IL18R1)		0.06	5	26
Interleukin-20 (IL20)		7.6	7	22
Interleukin-20 receptor subunit alpha (IL20RA)		1.9	6	22
Interleukin-22 receptor subunit alpha-1 (IL22RA1)		0.24	7	23
Interleukin-24 (IL24)		1.9	6	29

Interleukin-33 (IL33)		3.8	9	26
Latency-associated peptide transforming growth factor beta 1 (LAP TGFbeta1)		61	7	24
Leukemia inhibitory factor (LIF)		3.8	7	18
Leukemia inhibitory factor receptor (LIFR)		30.5	7	26
Macrophage colony-stimulating factor 1 (CSF1)		0.004	5	25
Macrophage inflammatory protein 1-alpha (CCL3)		0.06	6	14
Matrix metalloproteinase-1 (MMP1)	Interstitial collagenase	1.9	5	19
Matrix metalloproteinase-10 (MMP10)	Stromelysin-2	0.95	5	28
Monocyte chemotactic protein 1 (MCP1)	C-C motif chemokine 2 (CCL2)	0.03	6	13
Monocyte chemotactic protein 2 (MCP2)	C-C motif chemokine 8 (CCL8)	0.06	6	8

Monocyte chemotactic protein 3 (MCP3)	C-C motif chemokine 7 (CCL7)	0.48	7	17
Monocyte chemotactic protein 4 (MCP4)	C-C motif chemokine 13 (CCL13)	0.24	6	24
Natural killer cell receptor 2B4 (CD244)	SLAM family member 4	0.06	5	24
Neurotrophin-3 (NT3)	Hippocampus-derived neurotrophic factor (HDNF)	0.12	6	13
Neurturin (NRTN)		3.8	9	15
Oncostatin-M (OSM)		0.03	5	12
Osteoprotegerin (OPG)	Tumor necrosis factor receptor superfamily member 11B (TNFRSF11B)	0.24	6	12
Programmed cell death 1 ligand 1 (PD-L1)		3.8	9	25
Protein S100-A12 (EN-RAGE)	Extracellular newly identified RAGE-	122	8	17

	binding protein, Calgranulin C			
Receptor activator of nuclear factor kappa-B ligand	TNFSF11, TNF-related activation-induced cytokine (RANKL)	3.8	7	24
Signaling lymphocytic activation molecule (SLAMF1)		31	9	21
SIR2-like protein 2 (SIRT2)	NAD-dependent protein deacetylase sirtuin-2	7.6	8	22
STAM-binding protein (STAMPB)		7.6	5	27
Stem cell factor (SCF)	Kit ligand	1.9	5	20
Sulfotransferase 1A1 (ST1A1)	SULT1A1	244	6	25
T-cell surface glycoprotein CD5 (CD5)		0.06	5	22
T-cell surface glycoprotein CD6 isoform (CD6)		0.24	6	23

T-cell surface glycoprotein CD8 alpha chain (CD8A)		??	9	10
Thymic stromal lymphopoietin (TSLP)		3.8	6	20
TNF-beta (TNFB)	Lymphotoxin-alpha, Tumor necrosis factor ligand superfamily member 1	0.24	6	22
TNF-related apoptosis-inducing ligand (TRAIL)	Tumor necrosis factor ligand superfamily member 10 (TNFSF10)	0.95	5	17
Transforming growth factor alpha (TGF-alpha)	TGF type 1	0.48	6	27
TNF-related weak inducer of apoptosis (TWEAK)	Tumor necrosis factor (Ligand) superfamily, member 12 (TNFSF12)	1.9	6	11
Tumor necrosis factor (TNF)	TNF-alpha, Tumor necrosis factor	0.95	6	18

	ligand superfamily member 2			
Tumor necrosis factor ligand superfamily member 14 (TNFSF14)		0.95	6	15
Tumor necrosis factor receptor superfamily member 9 (TNFRSF9)		0.03	5	21
Urokinase-type plasminogen activator (uPA)		0.12	5	11
Vascular endothelial growth factor A (VEGFA)		0.06	6	8

593

594 Supplementary Table 2

595 Baseline characteristics of patients with AAD in relation to RAF

596

	AAD (n=43)	**AAD no RAF (n=20)**	**AAD RAF (n=23)**	**P-value (no RAF vs. RAF)**
Female sex, no. (%)	19 (44)	12 (60)	7 (30)	0.052
Age, years	40 ± 23	42 ± 12	39 ± 13	0.386
BMI, kg/m²	24.2 ± 2.9	24.3 ± 3.3	24.1 ± 2.7	0.873

Disease duration, years	8 [3-18]	14 [6-22]	5 [2-13]	0.010*
APS-2, no. (%)	20 (47)	10 (50)	10 (44)	0.669
Hydrocortisone equivalents, mg	20 [20-30]	20 [20-30]	25 [20-30]	0.195
Fludrocortisone, mg	0.1 [0.1-0.1]	0.1 [0.1-0.1]	0.1 [0.075-0.1]	0.229
Systolic BP, mmHg	120 ± 15	114 ± 14	122 ± 15	0.067
Diastolic BP, mmHg	76 ± 9	71 ± 9	75 ± 10	0.977
B-Hb, g/dL	14.3 ± 1.2	14.0 ± 1.3	14.7 ± 1.0	0.038*
B-HbA1c, mmol/mol	35 [32-36]	33.4 ± 3.8	33.7 ± 3.0	0.767
s-TSH, mIE/L	2.4 [1.5-3.7]	2.0 [1.1-3.6]	3.2 [2.0-3.8]	0.098
s-fT4, pmol/L	16 [15-19]	16.8 ± 2.8	16.5 ± 2.7	0.744
s-Creatinine, µmol/L	77 ± 12	75 [65-81]	76 [72-90]	0.273
eGFR, mL/min/L	93 [87-110]	94 [86-107]	90 [89-115]	0.587
s-Sodium, mmol/L	139 ± 3	140 [137-141]	139 [138-141]	0.750
s-Potassium, mmol/L	4.1 ± 0.4	4.0 ± 0.4	4.1 ± 0.3	0.488

s-Cholesterol, mmol/L	5.0 ± 0.8	5.1 ± 0.9	4.9 ± 0.8	0.501
s-HDL, mmol/L	1.4 ± 0.4	1.5 ± 0.4	1.3 ± 0.3	0.103
s-LDL, mmol/L	3.3 ± 0.8	3.2 ± 0.8	3.3 ± 0.8	0.545
s-Triglycerides, mmol/L	1.3 [1.0-1.5]	1.3 [1.0-1.8]	1.3 [1.1-1.5]	0.647
s-Cortisol, mmol/L	14 [1-52]	0.5 [0-5]	46 [22-111]	<0.0001*
p-ACTH, pmol/L	168 [70-278]	208 [53-278]	150 [70-225]	0.378

Abbreviations: AAD; autoimmune Addison's disease, APS-2; autoimmune polyendocrine syndrome type 2, B-Hb; blood hemoglobin, B-HbA1c; blood hemoglobin A1c, BP; blood pressure, BMI; body mass index, eGFR; estimated glomerular filtration rate, p-ACTH; plasma adrenocorticotropic hormone, RAF; residual adrenocortical function, s-fT4; serum free thyroxine, s-HDL; serum high-density lipoprotein, s-LDL; serum low-density lipoprotein, s-TSH; serum thyroid stimulating hormone.

Data are given as number (percentage, %), mean ±SD or median [IQR].

*P<0.050

*P<0.001

Supplementary Table 3

List of biological pathways and processes enriched for the 19 biomarkers significantly different between patients with AAD and healthy controls at baseline, obtained by STRING. The biological connections include biological processes by the Gene Ontology database (http://geneontology.org/docs/go-annotations/), functional pathways by KEGG pathways (https://www.genome.jp/kegg/pathway.html) and Reactome pathways (https://reactome.org/), and other diseases associated with similar biomarker networks by the disease-gene association database DISEASES (https://diseases.jensenlab.org/Search). The list is sorted by low-to-high significance (FDR 5%) and restricted to the ten processes for each biological connection category.

Biological processes (n=242) (Gene Ontology)				
Term ID	Pathway	Strength $Log10(\frac{observed}{expected})$	FDR	Proteins included in the network
GO:0070374	Positive regulation of erk1 and erk2 cascade	1.65	2.40e-09	CD4, MCP1, FGF23, XCL1, RAGE, GAL9, RANKL, RANK, FGF21
GO:0001934	Positive regulation of protein phosphorylation	1.12	2.91e-09	CD4, DR4, MCP1, IL12B, FGF23, DR5, XCL1, RAGE, GAL9, RANKL, IL6, RANK, FGF21
GO:1902533	Positive regulation of intracellular signal transduction	1.11	2.91e-09	CD4, DR4, MCP1, IL12B, FGF23, DR5, XCL1, RAGE, GAL9, RANKL, IL6, RANK, FGF21
GO:0009605	Response to external stimulus	0.85	3.27e-09	CD4, DR4, MCP1, IL12B, FGF23, DR5, XCL1, RAGE, GAL9, RANKL, IL6, ADM, MMP12, RANK, FGF21, SPON2
GO:0009967	Positive regulation of signal transduction	0.94	9.96e-09	CD4, DR4, MCP1, IL12B, FGF23, DR5, XCL1, RAGE, GAL9, RANKL, IL6, MMP12, RANK, FGF21
GO:0050870	Positive regulation of t cell activation	1.6	1.59e-08	CD4, MCP1, IL12B, XCL1, RAGE, GAL9, RANKL, IL6
GO:0043410	Positive regulation of mapk cascade	1.28	2.34e-08	CD4, MCP1, FGF23, XCL1, RAGE, GAL9, RANKL, IL6, RANK, FGF21
GO:0048584	Positive regulation of response to stimulus	0.84	2.34e-08	CD4, DR4, MCP1, IL12B, FGF23, DR5, XCL1, RAGE, GAL9, RANKL, IL6, MMP12, RANK, FGF21, SPON2
GO:0010033	Response to organic substance	0.74	4.44e-08	CD4, MCP1, PSGL1, IL12B, FGF23, XCL1, RAGE, GAL9, RANKL, IL6, ADM, MMP12, RANK, FGF21, TNFRSF9, SPON2
GO:0050900	Leukocyte migration	1.42	1.73e-07	DR4, MCP1, PSGL1, DR5, XCL1, RANKL, IL6, RANK

Molecular function (n=9) (Gene Ontology)				
GO:0048018	Receptor ligand activity	1.23	3.16e-05	MCP1, IL12B, FGF23, XCL1, RANKL, IL6, ADM, FGF21
GO:0005102	Signaling receptor binding	0.81	0.00046	CD4, MCP1, PSGL1, IL12B, FGF23, XCL1, RANKL, IL6, ADM, FGF21
GO:0005125	Cytokine activity	1.34	0.0017	MCP1, IL12B, XCL1, RANKL, IL6
GO:0005126	Cytokine receptor binding	1.29	0.0025	MCP1, IL12B, XCL1, RANKL, IL6
GO:0019955	Cytokine binding	1.49	0.0039	CD4, IL12B, RANK, TNFRSF9
GO:0070851	Growth factor receptor binding	1.47	0.0039	IL12B, FGF23, IL6, FGF21
GO:0045569	TRAIL binding	2.67	0.0067	DR4, DR5
GO:0005515	Protein binding	0.37	0.0070	CD4, DR4, MCP1, PSGL1, IL12B, FGF23, DR5, XCL1, RAGE, GAL9, RANKL, IL6, ADM, RANK, FGF21, TNFRSF9
GO:0005035	Death receptor activity	2.23	0.0237	DR4, RANK
KEGG pathways (n=19)				
hsa04060	Cytokine-cytokine receptor interaction	1.56	1.28e-11	CD4, DR4, MCP1, IL12B, DR5, XCL1, RANKL, IL6, RANK, TNFRSF9
hsa04061	Viral protein interaction with cytokine and cytokine receptor	1.73	6.09e-06	DR4, MCP1, DR5, XCL1, IL6
hsa05164	Influenza A	1.49	5.48e-05	DR4, MCP1, IL12B, DR5, IL6

hsa05323	Rheumatoid arthritis	1.69	0.00012	MCP1, RANKL, IL6, RANK
hsa04933	AGE-RAGE signaling pathway in diabetic complications	1.5	0.0082	MCP1, RAGE, IL6
hsa05142	Chagas disease	1.49	0.0082	MCP1, IL12B, IL6
hsa05135	Yersinia infection	1.39	0.0117	CD4, MCP1, IL6
hsa05224	Breast cancer	1.22	0.0157	FGF23, RANKL, FGF21
hsa05143	African trypanosomiasis	1.76	0.0229	IL12B, IL6
hsa05130	Pathogenic Escherichia coli infection	1.22	0.0261	DR4, DR5, IL6
Reactome pathways (n=11)				
HSA-1280215	Cytokine Signaling in Immune system	1.13	1.16e-05	CD4, MCP1, IL12B, RAGE, GAL9, RANKL, IL6, RANK, TNFRSF9
HSA-449147	Signaling by Interleukins	1.15	0.0031	CD4, MCP1, IL12B, RAGE, GAL9, IL6
HSA-6783783	Interleukin-10 signaling	1.84	0.0094	MCP1, IL12B, IL6
HSA-75158	TRAIL signaling	2.41	0.0199	DR4, DR5
HSA-3371378	Regulation by c-FLIP	2.27	0.0250	DR4, DR5
HSA-5218900	CASP8 activity is inhibited	2.27	0.0250	DR4, DR5

HSA-6803211	TP53 Regulates Transcription of Death Receptors and Ligands	2.27	0.0250	DR4, DR5
HSA-69416	Dimerization of procaspase-8	2.23	0.0250	DR4, DR5
HSA-5668541	TNFR2 non-canonical NF-kB pathway	1.5	0.0264	RANKL, RANK, TNFRSF9
HSA-5676594	TNF receptor superfamily (TNFSF) members mediating non-canonical NF-kB pathway	2.08	0.0273	RANKL, RANK
Disease-gene associations (n=20) (DISEASES)				
DOID:0050589	Inflammatory bowel disease	1.9	0.00076	CD4, MCP1, IL12B, IL6
DOID:65	Connective tissue disease	1.06	0.00076	CD4, DR4, MCP1, IL12B, FGF23, RANKL, IL6, RANK
DOID:0080001	Bone disease	3.01	0.0058	CD4, IL12B, FGF23, RANKL, IL6, RANK
DOID:403	Mouth disease	1.53	0.0058	CD4, FGF23, RANKL, IL6
DOID:417	Autoimmune disease	1.24	0.0058	CD4, DR4, MCP1, IL12B, IL6
DOID:77	Gastrointestinal system disease	1.08	0.0058	CD4, MCP1, IL12B, FGF23, RANKL, IL6

DOID:8632	Kaposis sarcoma	1.07	0.0058	CD4, IL6
DOID:0060032	Autoimmune disease of musculoskeletal system	1.38	0.0102	DR4, MCP1, IL12B, IL6
DOID:0080005	Bone remodeling disease	1.73	0.0112	FGF23, RANKL, RANK
DOID:0060039	Autoimmune disease of skin and connective tissue	2.41	0.0145	DR4, MCP1, IL12B

616

617

618

Supplementary Table 4

Biomarker differences between patients without and with RAF (P < 0.050) not corrected for multiple testing.

	AAD no RAF (n=19)	**AAD with RAF (n=23)**	**P value**
GH	9.61 [7.37-11.19]	7.15 [6.66-8.08]	0.007
THBS2	5.94 ± 0.15	5.82 ± 0.16	0.007
LEP	6.74 ± 0.71	5.75 ± 1.41	0.008
TGFB1	8.31 [8.0-8.82]	8.78 [8.32-9.43]	0.009
IL16	7.09 ± 0.58	7.51 ± 0.44	0.011
CD8A	6.42 ± 0.30	6.51 ± 0.34	0.014
IL24	1.76 ± 0.33	1.54 ± 0.29	0.024
BNP	1.06 [0.88-1.36]	1.27 [1.16-1.42]	0.025
AGRP	4.66 ± 0.46	4.96 ± 0.49	0.045

Data are given as mean ±SD or median [IQR].

Abbreviations: AAD; autoimmune Addison's disease, GRP; agouti-related peptide, BNP; brain natriuretic peptide, CD8A; Cluster of Differentiation 8a, GH; growth hormone, IL-16; interleukin 16, IL-24; interleukin 24, LEP; leptin, RAF; residual adrenocortical function. TGFB1; Transforming growth factor beta, THBS2; Thrombospondin 2.

**Statistically significant at P<0.050*

629 Supplementary Table 5

630

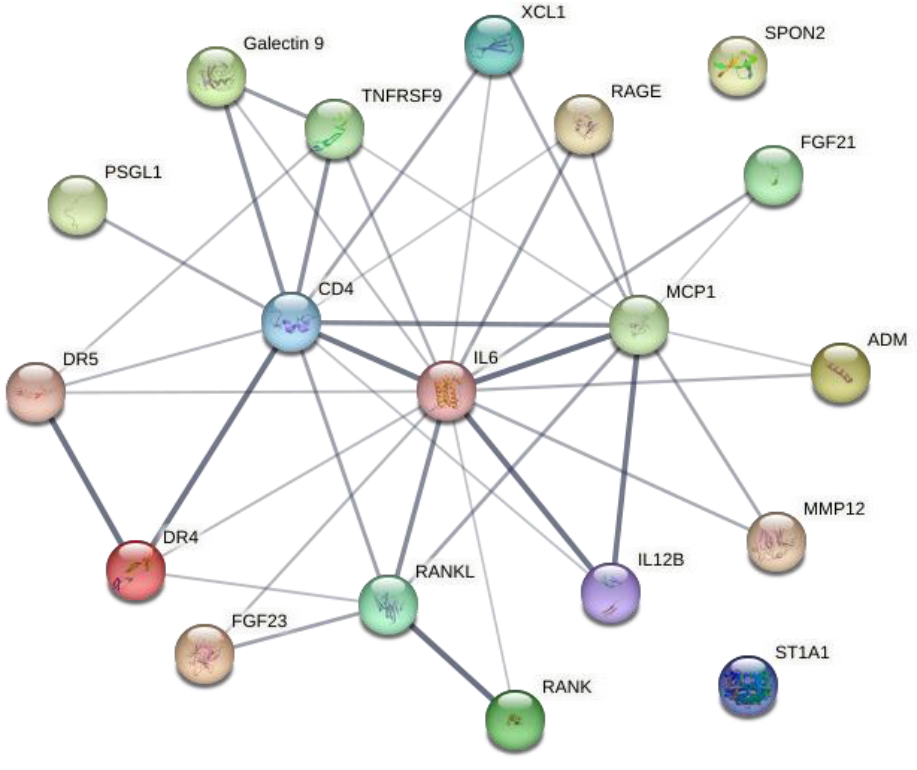
	Change in NPX	***P* value**
PDL2	-0,15	0.0001*
LEP	-0,25	0.0003*
FGF21	-0,31	0.002
MARCO	-0,16	0.002
DCN	-0,14	0.003
IL17D	-0,04	0.003
ACE2	-0,16	0.004
HAOX1	-0,32	0.004
PAR1	-0,21	0.004
GIF	-0,23	0.005
SORT1	-0,15	0.005
VSIG2	-0,19	0.005
GT	-0,48	0.006
IL7	0,29	0.009
VEGFD	-0,11	0.01
CD40l	-0,34	0.012
CTSL1	-0,15	0.014
MMP7	-0,13	0.014
MMP12	-0,20	0.015
lgGFcReceptor2B	-0,15	0.016

PRSS8	-0,15	0.016
FABP2	-0,35	0.018
THBS2	-0,05	0.018
TNFRSF9	-0,34	0.018
PAPPA	-0,12	0.019
DKK1	-0,01	0.021
CEACAM8	-0,40	0.022
PRELP	-0,12	0.022
TNFRSF11A	-0,19	0.023
PSGL1	-0,08	0.027
AMBP	-0,02	0.03
SERPINA12	-0,20	0.032
SOD2	-0,08	0.033
HSP27	-0,53	0.036
PTX3	-0,13	0.037
HOSCAR	-0,10	0.038
TNFRSF13B	-0,13	0.038
BNP	-0,16	0.044
IL1RA	-0,13	0.046

631 *Significant after correction for multiple testing

632 Supplementary Figure

633



Biological connection network including the 19 biomarkers significantly different between patients with AAD and healthy controls at baseline by STRING. For the full network of biomarkers, an estimation of the overall connection is given as an average clustering coefficient ranging from 0 to 1, where 0 indicates no connection and 1 a fully connected network. A significant network P-value indicates that the reported connections are not random. We employed the default interaction confidence score of 0.4 as a threshold for any biological connections between single biomarkers.

In the network figure, each biomarker is represented by a colored node, with connecting lines indicating similarities in biological function or structure. The thickness of the connecting line marks the strength of available evidence to support the biological connection.

The overall network of biomarkers was significantly enriched (*P* = 1.0e-16) with an average clustering coefficient of 0.721, suggesting *de facto* similarities in biological function or structure. The specific processes and pathways that were significantly enriched mainly related to cell signaling and immune response, including cytokine action and chemotaxis (Supplementary Table 3). Merging to the Disease-gene association database (DISEASE) showed that similar biomarker networks have been reported in other autoimmune diseases, diseases in bone and connective tissue, and infections, in particular (Supplementary Table 3).

Errata for
"Clues to Diagnosis and Clinical Outcomes in Autoimmune Addison's Disease"

Åse Bjorvatn Sævik

Thesis for the degree philosophiae doctor (PhD)
at the University of Bergen

01.10.23 ÅBS
(date and sign. of candidate)

02.10.23.
(date and sign. of faculty)

Errata

Page 9	Wrong words: “and sequential” – corrected to “tandem mass”
Page 9	Wrong word: "glycosylation” – corrected to “glycation”
Page 12	Updated status: “*In revision for Eur J Endocrinol*” updated to “A*ccepted in EJE in September 2023*” end the title extended to include “- a cross-sectional study”
Page 13	Updated status: “*In revision for J Clin Med*” updated to “*Accepted in J Clin Med in May 2023*”
Page 13	Updated status: “*In revision for Eur J Endocrinol*” updated to “*Accepted in EJE in June 2023*”
Page 30	Missing word: “to”
Page 31	Removal of word in the subheading: “And”S
Page 34	Wrong number: “21” corrected to “17”
Page 36	Misspelling: “years” corrected to "year”
Page 37	Missing comma inserted
Page 37	Duplicate wording: removed “were noted”
Page 43	Wrong word: "glycosylation” – corrected to “glycation”
Page 44	Inconsequent spelling: “sixty” corrected to "60”
Page 45	Misspelling: “deteriorate” corrected to "deteriorates”
Page 51	Misspelling: “PDL2” corrected to "PDL1”
Page 52, 55	Wrong word: “detectable” corrected to “quantifiable”
Page 53	Incomplete word: “there” corrected to "therefore”
Page 54	Misspelling: “pre-crisis” corrected to "pre-crises”
Page 62	Misspelling: "biomarkers” corrected to "biomarker”

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

uib.no

ISBN: 9788230845240 (print)
9788230849507 (PDF)