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Original Research

Altered biomarkers for cardiovascular disease and inflammation in autoimmune Addison's disease – a cross-sectional study

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Abstract

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

Design: Cross-sectional study.

Methods: We compared serum levels of 177 cardiovascular and inflammatory biomarkers in 43 patients with AAD at >18-h glucocorticoid withdrawal and 43 matched controls, overall and stratified for sex. Biomarker levels were correlated with the frequency of adrenal crises and quality of life (QoL) by AddiQoL-30. Finally, we investigated changes in biomarker levels following 250 µg tetracosactide injection in patients without residual adrenocortical function (RAF) to explore glucocorticoid-independent effects of high ACTH.

Results: Nineteen biomarkers significantly differed between patients with AAD and controls; all but 1 (ST1A1) were higher in AAD. Eight biomarkers were significantly higher in female patients compared with controls (IL6, MCP1, GAL9, SPON2, DR4, RAGE, TNFRSF9, and PGF), but none differed between male patients and controls. Levels of RAGE correlated with the frequency of adrenal crises ($r = 0.415$, $P = .006$) and AddiQoL-30 scores ($r = -0.347$, $P = .028$) but not after correction for multiple testing. PDL2 and leptin significantly declined 60 min after injection of ACTH in AAD without RAF (−0.15 normalized protein expression [NPX], $P = .0001$, and −0.25 NPX, $P = .0003$, respectively).

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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. High ACTH reduced PDL2 and leptin levels in a glucocorticoid-independent manner but the overall effect on biomarker profiles was small.

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

Significance

Cardiovascular health seems to be impaired in AAD, but which patients carry higher risk and why is not fully known. We show that biomarkers of CVD and inflammation are altered in AAD, with 18 biomarkers elevated and one reduced in AAD compared with controls. Eight biomarkers differed between female patients and controls but none between male patients and controls. Higher RAGE levels were associated with more adrenal crisis and lower QoL. High ACTH exposure reduced PDL2 and leptin levels in a glucocorticoid-independent manner. Our results indicate sex-specific differences in cardiovascular and inflammatory biomarkers, and RAGE as an exploratory marker of disease severity in AAD, and some glucocorticoid-independent effects of high ACTH on biomarker levels.

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 more cardiovascular disease (CVD).[1] Swedish population-based studies have demonstrated higher prescription rates for CVD medications in patients with AAD, and increased risk of ischaemic 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 dyslipidaemia 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. The inability of conventional GC replacement to replicate the physiological fluctuations in cortisol levels is commonly blamed for the unfavourable cardiovascular outcomes in adrenal insufficiency.[5,6] Factors beyond GC replacement likely play important roles for cardiovascular health as well but are less explored.[7-10]

Rarely investigated in AAD, the autoimmune aetiology 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, the 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 se*.[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 suggested associated with impaired patient well-being 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 and to assess whether altered levels of inflammatory markers can be linked with clinical outcomes in AAD, as suggested for a broad range of other conditions.[18]

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.[19] By promiscuous binding to the full range of melanocortin receptors (MC1-5R), ACTH might in theory modulate cardiovascular risk and inflammation in AAD.[20] 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 disease-specific QoL, as well as any GC-independent impact of 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 and 43 healthy controls matched for sex, age (in decade), and body mass index (BMI, ±1 kg/m^2). The inclusion criteria for patients and controls have previously been reported in detail.[11,21] In short, all included patients had confirmed AAD with proven 21-hydroxylase autoantibodies and chronic use of GC replacement and (except for 2 female patients) MC replacement therapy. The median hydrocortisone equivalent dose was 20 mg (20-30). All patients used immediate-release formulations and followed a once (3%), twice (63%), or 3 times (34%) daily dosing regimen. The median fludrocortisone dose was 0.1 mg (0.1-0.1). Five female patients used dehydroepiandrosterone replacement as well (median 25 mg [12.5-25.0]), but this was paused for at least 1 week before the blood sampling. Twenty-three patients had confirmed RAF, defined as serum cortisol (serum cortisol [>0.914 nmol/L] and 11-deoxycortisol [>0.114 nmol/L]) in a morning blood sample. None of the controls had any known autoimmune disease, and neither patients nor controls had a history of any cardiovascular event or diabetes mellitus. A 57-year-old female patient and a 68-year-old male patient

were prescribed angiotensin II inhibitors, of which the male patient was prescribed statins as well. Six female patients with AAD and 2 female controls used oral contraceptive pills.

Noted patient characteristics included any autoimmune comorbidity, patient-reported number of adrenal crises the past year, and QoL by the disease-specific AddiQoL-30 questionnaire. An adrenal crisis was defined to require acute hospital admission and intravenous hydrocortisone and fluid therapy.

Baseline blood samples were obtained on an agreed morning, and the patients with AAD had abstained from any GC and MC replacement for at least 18 and 24 h, respectively, before sampling. All participants were non-fasting as an extra safety measure for patients upon GC and MC withdrawal. Morning ACTH stimulation tests were performed in both patients and controls with intravenous injection of synthetic $ACTH_{1-24}$ (250 µg tetracosactide, Synacthen®). We analysed serum samples collected before and 60 min after $ACTH_{1-24}$ 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 the coefficient of variance (CV%), lower limit of detection (LOD), and biomarker synonyms is included in Table S1. The biomarkers were analysed by proximity extension assay (PEA) technology, which combines dual-recognition immunoassay and quantitative polymerase chain reaction, yielding improved assay specificity and multiplexing capacity.[22,23] The results are given as normalized protein expression (NPX), which is an arbitrary unit on a $\log_2$ scale calculated from normalized Ct values. Thus, NPX may only be used for relative quantification, and an increase of 1 unit (1 NPX) represents a doubling in protein concentration.[24,25] Biomarker values below LOD were included, as suggested by Olink.[26] Initially, 44 patients and 44 controls were included, but 1 patient sample failed to pass the initial quality control by Olink and was therefore excluded from the study together with its respective control before any statistical analyses.

String

Any biological connections, ie, similarities in functions or structure, for significant biomarkers were mapped by biomarker connection networks using the online database Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; version 11.5).[27]

Statistics

Descriptive statistics are presented as percentage, mean (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-sample *t*-test were used to investigate any significant change in biomarker values before and 60 min 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 the 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 $P < .05$, and multiple testing was corrected for by the Benjamini–Hochberg method using a false discovery rate (FDR) of 5% except for when assessing baseline characteristics.

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). The study was conducted in agreement with the Declaration of Helsinki (2013 version) and the principles of good clinical practice (CPMP/ICH/135/95). 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 Tables S2 and S3. There were no significant differences in the 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/m^2 ± 2.3 vs 25.1 kg/m^2 ± 3.1, $P = .022$, and 23.3 kg/m^2 ± 2.2 vs 25.2 kg/m^2 ± 3.2, $P = .031$, respectively), but there were no significant differences in age (42 years ± 11 vs 39 years ± 11, $P = .462$, and 41 years ± 11 vs 40 years ± 11, $P = .722$, respectively). Forty-seven per cent of the patients had at least 1 concomitant autoimmune disease (autoimmune hypothyroidism in all), in the following denoted as autoimmune polyendocrine syndrome type 2 (APS2).

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), tumour 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, $P = .004$) (Figures 1 and 2). Any biological connections between the 19 biomarkers are depicted in Table S4 and supplementary figure.

Three out of the 19 biomarkers correlated with the number of adrenal crises: RAGE ($r = 0.415$, $P = .006$), CD4 ($r = 0.338$, $P = .029$), and FGF21 ($r = -0.317$, $P = .041$). RAGE also

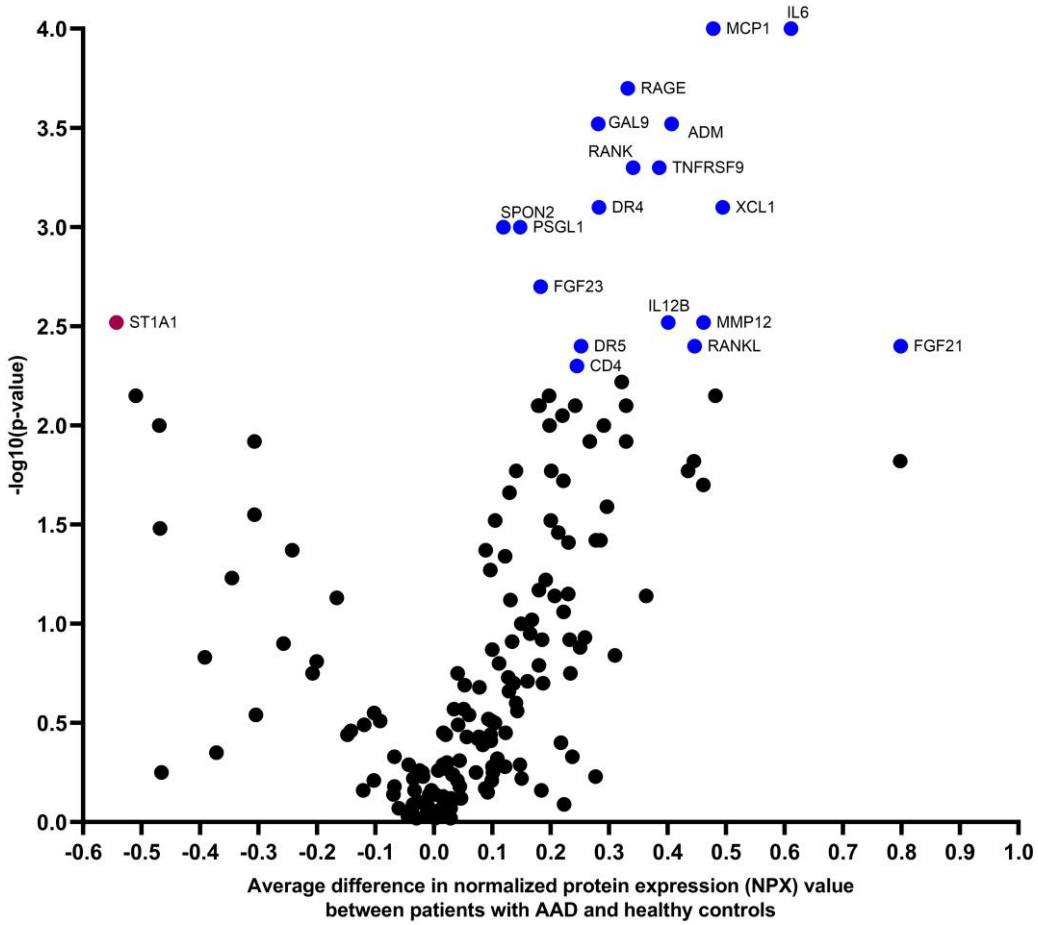

Figure 1. Volcano plot depicting differences in average normalized protein expression (NPX) values between patients with AAD and healthy controls for the 177 analysed biomarkers, of which 18 were significantly higher (labelled positive values, to the right) and 1 significantly lower (labelled negative value, to the left) in patients compared with control. Unlabelled dots represent biomarkers that were not significantly different between patients and controls. Abbreviations are given for all the significant biomarkers.

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	.8
BMI, kg/m^2	24.2 ± 2.9	24.3 ± 2.9	.8

Data are given as number (percentage %) and mean ± SD.
Abbreviations: AAD, autoimmune Addison's disease; BMI, body mass index; no., number.

negatively correlated with AddiQoL-30 scores ($r = -0.347$, $P = .028$) (Table 2). The correlations were not statistically significant when corrected for multiple testing (FDR 5%).

Stratifying biomarker comparison 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 3), but no significant differences in biomarker levels were found between male patients and controls (data not shown).

When comparing women and men with AAD, more women (63%) than men (33%) had APS2 but the difference did not reach statistical significance ($P = .052$). Comparisons of other baseline characteristics between women and men with AAD are given in Table S2. Levels of leptin (LEP) and growth hormone (GH) were significantly higher in female patients compared with male patients and in female controls compared with male controls (LEP: 7.1 NPX ± 1.1 vs 5.4 NPX ± 0.6, $P < .0001$, and 6.9 NPX ± 0.9 vs 5.4 NPX ± 1.3, $P < .0001$, respectively; GH: 9.9 NPX ± 1.9 vs 7.1 NPX ± 1.5, $P < .0001$, and 9.8 NPX ± 2.1 vs 7.6 NPX ± 1.3, $P = .0002$, respectively).

No significant differences in biomarker levels were found between patients with and without RAF (Table S5).

In AAD without RAF ($n = 19$), there was a significant reduction in programmed death-ligand 2 (PDL2) (−0.15 NPX, $P = .0001$) and LEP (−0.25 NPX, $P = .0003$) 60 min after injection of $ACTH_{1-24}$ (Table S6).

Discussion

We identified 19 cardiovascular and inflammatory biomarkers that differed between patients with AAD and healthy controls. All but 1 biomarker were elevated in patients, indicating an unfavourable cardiovascular milieu and proinflammatory state at the molecular level, with the greatest difference noted for FGF21. Alterations in biomarker profiles were sex specific, with 8 biomarkers differing between female patients and controls but none between male patients and controls. Any

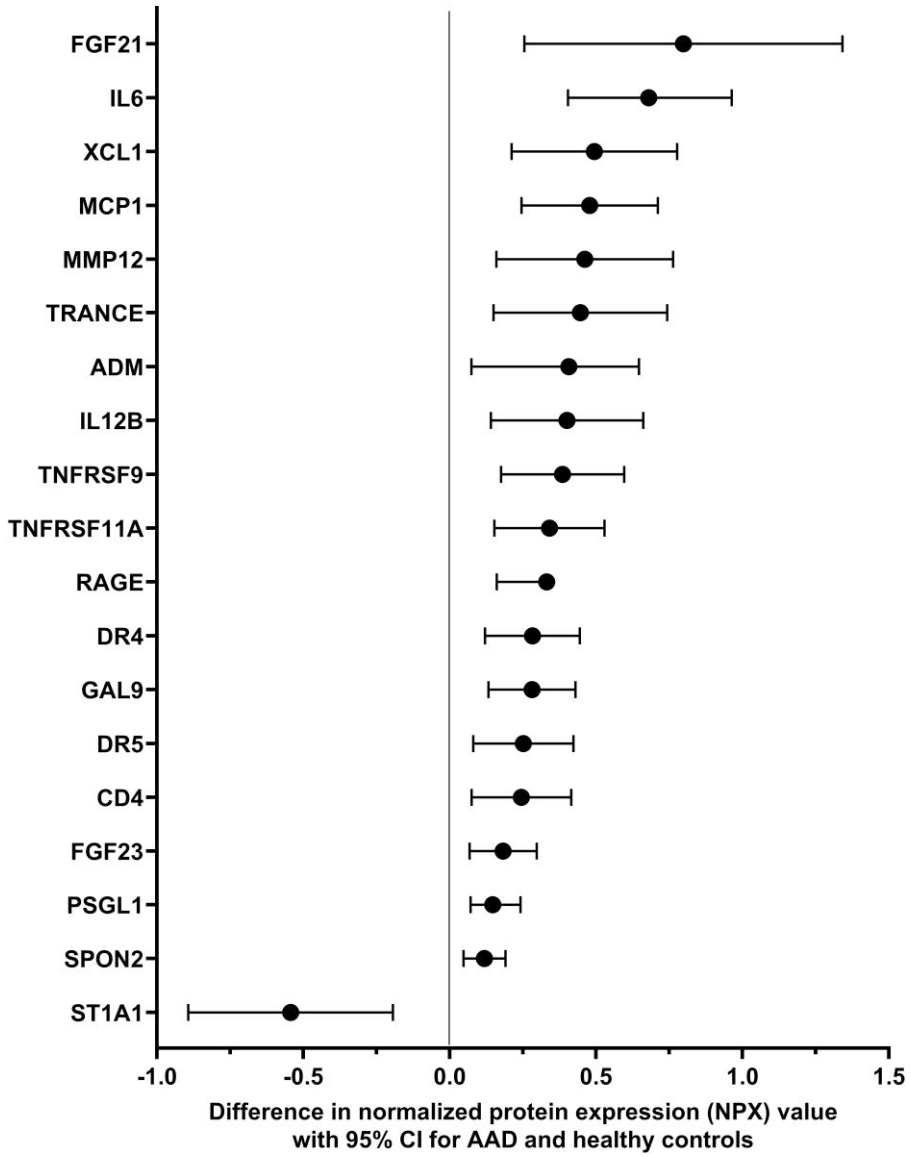

Figure 2. Forest plot depicting differences in average normalized protein expression (NPX) values with 95% confidence interval (95% CI) between 19 biomarkers significantly different between patients with AAD and healthy controls.

clinical relevance of altered biomarker levels remains uncertain, but RAGE could be an exploratory marker of disease severity in AAD, with higher levels linked to more adrenal crises and reduced QoL. We further demonstrate a decline in PDL2 and LEP levels following high ACTH exposure 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,[28] FGF21 has recently been implicated in the development and renewal of adrenocortical tissue.[29] Even in the setting of adrenal insufficiency, injection of recombinant FGF21 is reported to stimulate cortisol secretion and lower ACTH in mice.[30] Hence, elevated FGF21 could represent a compensatory attempt to keep up GC production as adrenocortical tissue is progressively lost. A recent meta-analysis found FGF21 to be an independent predictor of cardiometabolic disease progression and even death.[31] On the other hand, FGF21 has been shown to have cardioprotective effects with promising therapeutic potential in cardiometabolic diseases.[28] Taken together, studies on longitudinal associations between FGF21 levels and cardiometabolic outcomes in patients with AAD are needed.

Elevated IL6 seems to be a consistent finding in primary adrenal insufficiency (PAI), regardless of any recent intake of hydrocortisone replacement[32,33] or not.[33] This might imply that factors beyond GC exposure contribute to raise IL6 levels in AAD. The link between IL6 and CVD risk is otherwise well described, with mounting evidence from large-scale human studies even pointing to causality.[34,35] IL6 exerts a wide range of proinflammatory and immunoregulatory actions and is proposed as a therapeutic target in a multitude of diseases, including autoimmunity.[36] In refractory rheumatoid arthritis, IL6 receptor inhibition may alleviate symptoms and reduce disease activity but at the cost of increased susceptibility to infections.[36] 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.[36,37]

Table 2. 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. of AC past year	*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
	P	.938	.029	.041	.902	.059	.115	.923	.702	.386	.101	.006	.603	.941	.972	.232	.766	.465	.939	.206
	n	42	42	42	43	42	43	42	43	42	42	42	43	42	43	43	42	42	42	42
AddiQol-30	*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
	P	.227	.328	.801	.083	.855	.212	.755	.185	.708	.581	.028	.228	.25	.144	.242	.416	.629	.229	.53
	n	40	40	40	41	40	41	40	41	40	40	40	41	40	41	41	40	40	40	40

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

*$P < .050$.

Table 3. Biomarkers significantly different between female patients with AAD and female healthy controls.

Biomarker	Female AAD ($n = 19$)	Female HC ($n = 19$)	*P* value
IL6	3.8 (3.6-4.6)	3.2 (2.6-3.4)	<.0001
MCP1	13.3 ± 0.4	12.6 ± 0.6	<.0001
GAL9	8.3 ± 0.3	7.9 ± 0.3	.0002
SPON2	9.0 ± 0.1	8.9 ± 0.2	.0005
DR4	3.3 ± 0.4	2.9 ± 0.3	.0008
PGF	8.0 ± 0.4	7.6 ± 0.3	.001
RAGE	14.1 ± 0.4	13.5 ± 0.3	.001
TNFRSF9	7.4 ± 0.5	7.2 ± 0.4	.002

Data are given as mean ± SD or median (IQR).
Abbreviations: AAD, autoimmune Addison's disease; DR4, death receptor 4; GAL9, galectin 9; HC, healthy controls; IL6, interleukin 6; MCP1, monocyte chemoattractant protein 1; PGF, placental growth factor; RAGE, receptor for advanced glycosylation end product; SPON2, spondin 2; TNFRSF9, tumour necrosis factor receptor superfamily member 9.

Our finding of elevated MCP1 is in line with a recent report on 15 patients with AAD,[38] but the clinical implications of this remain to be explored. Available literature suggests that MCP1 takes part in the pathophysiology of several autoimmune diseases as well as CVD, with experimental models showing tapered inflammation in, for example, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis and reduced atherosclerotic plaque formation following inhibition of MCP1 or its receptor, CCR2.[39] Outcomes in human trials, however, have been mixed and occasionally detrimental, fuelling the debate as to whether MCP1 is mainly a friend or foe.[40]

Named for its role as a receptor for advanced glycosylation end products (AGEs), RAGE is best known as a mediator of vascular inflammation and endothelial dysfunction, linked to the development of atherosclerosis in general and diabetic complications in particular.[41] We were intrigued to find the medium–strong correlations between RAGE levels and frequency of adrenal crises and AddiQoL-30 scores in AAD. Importantly, the correlation did not remain statistically significant after correction for multiple testing, which calls for cautious emphasis of RAGE as a future marker of disease severity in AAD.

Other biomarkers that differed between patients with AAD and healthy controls included mediators of apoptosis (GAL9, DR4, and DR5[42,43]), inflammatory agents (TNFRSF9, XCL1, CD4, and IL12b[42,44-46]), regulators of bone turnover (RANK, RANKL, and FGF23[47]), a hypotensive peptide (ADM[48]), and proteins involved in general physiology, ie, tissue remodelling (MMP12[49]), cell adhesion (PSGL1 and SPON2[50,51]), and sulphation (ST1A1[52]).

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, 8 biomarkers were higher in patients compared with controls, both for AAD overall and for the subgroup of women. In contrast, there were no significant differences 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 LEP and GH levels in women compared with men, in both patients and controls, corresponds to established sex differences in the concentrations of these hormones.[53,54] 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[54] but not LEP as levels positively correlate with BMI.[53]

Biomarker levels did not differ between patients with and without RAF, possibly due to insufficient sample size and the inclusion of very low levels of residual GC production. 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 (eg, rheumatoid arthritis, gout, psoriasis, and ulcerative colitis).[55] For long, the anti-inflammatory effect was considered the mere result of GC induction, until 2002 when Getting *et al.*[56] demonstrated preserved anti-inflammatory effects of ACTH after adrenalectomy. In the present study, the $ACTH_{1-24}$ stimulation test allowed for suis-generating exploration of direct *in vivo* effects of high ACTH, but except for significant reductions in PDL2 and LEP levels, we found limited effects of high ACTH on the cardiovascular and inflammatory biomarker levels in AAD without RAF.

To the best of our knowledge, this is nevertheless 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 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.[57] We have not been able to find any publications successfully linking PDL2 to any autoimmune endocrinopathy.

Mainly secreted by adipocytes, LEP conveys information on energy stores to the brain and regulates appetite by acting on the melanocortin system and the hypothalamic-pituitary-adrenal axis. In turn, LEP secretion is stimulated by GC and inhibited by ACTH.[58,59] The latter was nicely depicted in the present study, as LEP significantly decreased following high ACTH exposure in patients without RAF. There was a non-significant tendency towards higher LEP in AAD compared with controls at baseline, corresponding to a previous report on 63 patients with AAD.[60] Taken together, we speculate that the observed decrease in LEP 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 that the contrasting findings are due to differences in GC exposure,[61,62] as blood samples in the previous study were collected after patients had taken their morning GC replacement[32] whereas patients here had abstained from any GC replacement for at least 18 h prior to sampling. In addition, the previous study included participants with metabolic syndrome and diabetes mellitus (types 1 and 2), significantly overrepresented in patients compared with controls, which could potentially have affected the results.[32]

Strengths of the present study include a well-characterized patient cohort of autoimmune AD aetiology only, without concomitant DM, metabolic syndrome, or overt CVD, and comparing biomarker profiles with matched controls. Another strength is the standardization of blood sampling with regard to time of the day (morning) and GC exposure (withdrawal) in patients. Taking the recently recognized phenomenon of RAF into account further 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 for the prediction of future cardiovascular events or assessing any temporal associations between cortisol fluctuations throughout the day and changes in biomarker levels. As a third point, matching can never be done perfectly, here exemplified by oral contraceptive pills used by more female patients ($n = 6$) than female controls ($n = 2$), but excluding these women gave similar biomarker results. Otherwise, 2 patients were prescribed angiotensin II inhibitors of which 1 was prescribed statins as well, but excluding these patients did not alter the results. We are aware that the numbers of adrenal crises might be inaccurate, as they were based on patients' reports and not cross-checked against their medical journals. Even though the criteria for an adrenal crisis were acute hospital admission and intravenous hydrocortisone and fluid therapy, the reported numbers might in some cases reflect subjective precrisis[63] or be erroneously low due to recollection bias. 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 before the $ACTH_{1-24}$ stimulation testing. As the biomarker half-lives are largely unknown, it is also possible that 60 min was too short to detect all true changes following the injection of ACTH. Otherwise, the injected dose of 250 µg tetracosactide constitute a far supraphysiological ACTH exposure,[64] further questioning any clinical relevance of the observed decline in PDL2 and LEP levels. 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 analysed markers are reported to change following eating, including a reduction in LEP.[65] Finally, several biomarkers were nearly significant when correcting for multiple testing, and interesting connections may have been erroneously overlooked.

To conclude, patients with AAD and especially women have increased levels of cardiovascular and inflammatory biomarker profiles compared with controls, with the greatest difference found for FGF21 levels. Future work is needed to determine any clinical relevance of the altered biomarkers, including the exploratory link between higher levels of RAGE and more adrenal crises and reduced QoL in AAD. High ACTH exposure seems to reduce PDL2 and LEP in a GC-independent manner, but the overall impact of elevated ACTH on cardiovascular health and inflammation in AAD remains to be determined.

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Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

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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.

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