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Original Research

Substantial changes in inflammatory and cardiovascular biomarkers in patients with autonomous cortisol secretion

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Abstract

Objective: To map inflammatory biomarkers in patients with autonomous cortisol secretion (ACS) and overt Cushing syndrome (CS).

Method: Observational study including serum from prospectively included patients with ACS ($n = 63$), adrenal CS ($n = 2$), pituitary CS ($n = 8$), and healthy subjects ($n = 120$). Serum samples were analysed for 92 inflammatory biomarkers using proximity extension assay (OLINK).

Results: Combined, the ACS and CS patients displayed significant differences in levels of 49/92 inflammatory biomarkers (46 increased/3 decreased) compared with healthy controls. No differences in biomarker levels were found between ACS and overt CS, and none of the biomarkers correlated with the degree of hypercortisolism. Postoperative samples were available for 17 patients, median 24 months (range 6–40) after surgery and biochemical curation. There was no significant normalization of the biomarkers postoperatively.

Conclusion: There was a systemic rise in inflammatory biomarkers in patients with ACS and CS, not correlated to the degree of hypercortisolism. These biomarkers were not normalized following biochemical cure.

Keywords: autonomous cortisol secretion, Cushing syndrome, proteomics, inflammation, cardiovascular biomarkers

Significance

Autonomous cortisol secretion (ACS) is common, with a prevalence of 1%–2% in the adult population. The condition is associated with the metabolic syndrome, which disposes for cardiovascular disease. In this prospective observational study, we found a broad increase in inflammatory biomarkers among patients with ACS and Cushing syndrome compared with healthy subjects. Inflammatory markers related to diabetes, hypertension, osteoporosis, and cardiovascular disease were elevated. The degree of inflammation did not correlate with the degree of hypercortisolism and did not normalize after biochemical cure. Our data provide novel insights into the inflammatory response in ACS patients and indicate a persistent cardiovascular and metabolic burden, despite successful cure of hypercortisolism.

Introduction

With the widespread use of computed tomography (CT) and magnetic resonance imaging (MRI) scans, incidental adrenal tumours are commonly detected. Up to 5% of the adult population have adrenal incidentalomas. The prevalence is higher in the elderly population and in patients with type 2 diabetes, hypertension, and obesity.[1-5] Even though the majority of adrenal incidentalomas is non-secreting, 20%–50% show some degree of excess cortisol production,[6-8] known as autonomous cortisol secretion (ACS).[9]

The symptomatology in patients with ACS is heterogeneous, and the condition is associated with the metabolic syndrome[8] and mental and psychiatric problems.[10] Previous studies have shown increased prevalence of type 2 diabetes, hypertension,[6,11-14] obesity, and hypercholesterolaemia in patients with ACS.[6,15] The unfavourable metabolic profile causes an increased risk of cardiovascular events[6,11,12,16] and mortality.[11,17,18] ACS also has a negative impact on bone, with increased prevalence of osteoporosis and osteoporotic fractures.[19]

Whether adrenalectomy attenuates the cardiovascular risk in ACS is debated. Some studies report improvement in metabolic parameters,[20-23] while others do not.[24,25] Prospective studies with long-term follow-up and clinical end-points, such as cardiovascular events and mortality, are still lacking. This knowledge gap is reflected in the fact that guidelines for the treatment of ACS take into consideration many factors and encourage individualization, which could lead to unwanted variation within the health services.[9]

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Overt Cushing syndrome (CS) is associated with systemic inflammation. Several studies have demonstrated elevated C-reactive protein, interleukins (IL), and cytokines in patients with CS,[26-29] and this inflammatory state persists years after biochemical cure.[29] Whether this is also the case for ACS has not been studied. We here investigate a panel of 92 inflammatory and cardiovascular biomarkers in ACS compared with overt CS and healthy controls.

Methods

Study organization and participants

From June 2016 to June 2021, we consecutively included patients with adrenal incidentalomas diagnosed with ACS and adrenal CS and patients diagnosed with pituitary CS, in a prospective observational study at Haukeland University Hospital, Bergen, Norway ($n = 73$). Healthy controls ($n = 120$) were recruited from the hospital and university staff. Relevant laboratory and clinical data were obtained at enrolment. Seventeen patients with ACS and CS successfully treated with adrenalectomy or pituitary surgery were sampled at least 6 months after having tapered off cortisone treatment. Fifty-six patients had only preoperative samples and four only postoperative samples. In four patients, two preoperative samples were available and used for reproducibility assessment of the biomarkers. None of the healthy controls or the patients was on oral oestrogen therapy, and none of our patients had taken licorice, grape fruit juice, or St Johannes herb the last week before biochemical testing. Low dexamethasone bioavailability as a cause of false positive dexamethasone suppression test (DST) was excluded by the measurement of serum dexamethasone.[30]

Definitions

ACS was defined as a morning cortisol level (before 9 AM) following 1 mg overnight DST above 50 nmol/L, with the group suppressing cortisol between 50 and 138 nmol/L termed possible ACS. Overt CS was defined according to European guidelines for diagnostics of CS, with cortisol following DST above 50 nmol/L, 24 h urine free cortisol (UFC) above upper normal range for the assay (165 nmol/L/24 h at our laboratory), and a typical clinical picture.[31] Postoperative biochemical cure was defined as a cortisol level post-DST below 50 nmol/L, 24 h UFC below 165 nmol/L/24 h, and adrenocorticotrophic (ACTH) in the normal reference range.

ACS/CS patients were compared with a group of 120 healthy individuals, covering both sexes and a large age and body mass index (BMI) range. Since there is insufficient information on the impact of age, sex, and BMI on the biomarkers, the healthy controls were used to calculate each biomarker's correlation to age, sex, and BMI and thus determine the need for partitioning. All study subjects were also categorized into age groups: 18-39 years, 40-59 years, and ≥60 years.

Assay of hormones

All hormone analyses were performed at the Hormone Laboratory at Haukeland University Hospital. Serum cortisol and dexamethasone were assayed using an in-house developed high-performance liquid chromatography tandem mass spectrometry (LCMS/MS).[32] For cortisol, the reference range was 120–600 nmol/L for samples drawn before 10:00 AM The assay precision was 4.5%–7.4% relative standard deviations (RSD), and the accuracy ranged from 97% to 101%.[32] ACTH was determined by chemiluminescent immunoassay (Immulite 2000 XPI, Siemens Healthineers, Erlangen, Germany). The lower limit of quantification for ACTH was 1.1 pmol/L. ACTH had a total RSD of 5.0% at 3 pmol/L and 8.4% at 49 pmol/L. Urine samples were collected over a period of 24 h and the volume was noted. Urinary cortisol was analysed by LCMS/MS with an analytical precision of RSD 10% at a 140 nmol/L concentration. Free cortisol excretion above 165 nmol/24 h in at least two separate samples was required to diagnose CS, according to clinical guidelines.[31]

Assay of inflammatory and cardiovascular biomarkers

Serum samples (40 mL) were collected from all subjects and stored at −80 °C until biomarker analyses. The panel consisted of 92 inflammatory and cardiovascular biomarkers (Proseek Multiplex Inflammation I Panel), Olink Bioscience (Uppsala, Sweden). Briefly, the biomarkers were analysed simultaneously using multiplex proximity extension technique. Oligonucleotide-labelled antibody probe pairs were bound to the respective target protein, followed by a proximity-dependent DNA polymerization step that provided a reporter sequence that was measured by quantitative PCR.[33] The proximity extension assays generated quantification cycle values (NPX values), and the data were normalized for both intra- and inter-plate variation and applied a fixed correction factor. The results are reported in log2 scale where an increase of one NPX unit corresponded to a doubling of the protein concentration. Details on the limit of detection (LOD) and RSD for all biomarkers are provided in Table S1.

Statistical analyses

Data were analysed using the Statistical Package for the Social Sciences (SPSS Version 26.0; IBM Corporation, Armonk, NY, USA). Median, range, and percent were used to describe the cohorts. Student's *t*-test, Mann–Whitney *U* test, and Wilcoxon signed rank test were used to detect differences in biomarkers between groups as appropriate. Benjamin Hochberg procedure for adjustment of *P* value was used to correct for possible false discovery rate (FDR) due to multiple comparisons. NPX values below LOD were set to LOD. The normal range for each biomarker was defined based on the 2.5% and 97.5% of healthy subjects while applying Dixon's criteria to exclude outliers.[34] Harris and Boyd's criteria were used to assess the need for dividing reference intervals into subgroups according to sex and age.[35] Spearman's rho was used to map the correlations in healthy controls between biomarkers and age, BMI, and sex, which possibly could affect the results. When correlation was found, linear regression was used to adjust for the independent variables age, sex, and BMI (the biomarker data were set as the dependent variable). Linear regression was also used to correct for smoking habits for all significant biomarkers. A two-tailed *P* value <.05 was considered statistically significant. Reproducibility was assessed by Mann–Whitney *U* test for all relevant biomarkers in the four patients that had provided preoperative samples at two separate occasions, although the small sample size hindered further analysis of reproducibility such as intraclass coefficient.

Table 1. Demographics data for healthy controls and patients.

	Healthy controls		ACS/adrenal CS patients		Pituitary CS patients	
	($n = 120$)		($n = 65$)		($n = 8$)	
Women, *n* (%)	65	(54.2)	48	(73.8)	8	(100)
Age, median years (range)	40	(23–68)	63	(40–80)	59	(34–72)
BMI, median kg/m^2 (range)	23.9	(15.8–33.6)	26.8	(19.2–40.3)	27.3	(21.0–40.4)
Smokers, *n* (%)	9	(7.5)	24	(37)	7	(78)
Drinking alcohol, *n* (%)[a]	105	(87.5)	56	(86)	7	(78)
Hypertension, *n* (%)	0	(0%)	23	(35.4)	5	(56)
Diabetes type 2, *n* (%)	0	(0%)	9	(13.8)	1	(11)
Treatment for dyslipidaemia, *n* (%)	0	(0%)	18	(21.7)	2	(21)
Osteoporosis, *n* (%)	0	(0%)	10	(15.4)	3	(33)
Unilateral AI, *n* (%)		NA	52	(71.2)		NR
Size of unilateral AI, median mm (range)		NA	24	(10–51)		NR
Bilateral AI, *n* (%)		NA	21	(28.8)		NR
Size of largest AI if bilateral, median mm (range)		NA	33	(21–42)		NR
Serum creatinine, umol/L		NA	69.5	(44–130)	73.0	(53–103)
Estimated GFR, mL/min/1.73 m^2		NA	78.5	(35–106)	76.0	(60–114)
Basal cortisol, median nmol/L (range)		NA	380	(168–890)	477	(334–758)
Morning ACTH, median pmol/L (range)		NA	1.8	(<1.1–4.4)	10.5	(2.4–24)
Cortisol after 1 mg DST, median nmol/L (range)		NA	101	(64–486)	307	(49–458)[b]
Late night saliva cortisol, median nmol/L (range)		NA	1.9	(0.6–3.2)	4.1	(3.4–72)
DHEAS, median µmol/L (range)		NA	0.88	(<0.4–4.5)	NA	
24 h UFC, median nmol/24t (range)		NA	81	(13–321)[c]	379	(23–1056)

Hypertension was defined as receiving anti-hypertensive treatment or having a blood pressure above 140/90 at the day of sample collection.
Dyslipidaemia was defined as receiving lipid-lowering drugs.
All patients had performed a morning serum cortisol, serum DHEAS and plasma ACTH measurement, a DST, two late night saliva cortisol measurements, and at least one 24 h UFC measurement.
Reference ranges for biochemical tests: basal serum cortisol (60–600 nmol/L), morning plasma ACTH (<10.2 pmol/L), cortisol after DST (<50 nmol/L), late night saliva cortisol (<2.8 nmol/L), DHEAS (2.0–10.5 µmol/L), and 24 h UFC (<165 nmol/24 h).
Abbreviations: AI, adrenal incidentaloma; DHEAS, dehydroepiandrostendione sulphate; NA, information not available; and NR, information not relevant.
[a]Drinking alcohol once a week or more.
[b]One patient with overt pituitary CS had post-DST cortisol below 50 nmol/L. She had clinical CS, several elevated saliva cortisol measurements, and UFC above 1000 nmol/24 h. She also had a pituitary tumour and has been cured for CS after surgery.
[c]One patient from the ACS group and one from the pituitary CS group had very low 24 h UFC. The ACS patients had severe kidney failure, which can explain the low UFC level, and there was no doubt about their diagnosis based on other diagnostic tests.

Ethics

The Regional Committee for Medical and Health Ethics of Western Norway approved the study protocol, and all participants gave their written informed consent (REK no. 2014/2170). The research was conducted according to the Declaration of Helsinki.

Results

Subject characteristics

The clinical characteristics of patients and healthy controls are shown in Table 1. The proportion of men and women was equal between the two groups. The median age and BMI were lower in the healthy control group than in the ACS/CS group. In addition, smoking was more common among patients.

A total of 63 patients with ACS were included, of whom 36 (55%) had possible ACS and 27 (42%) patients had ACS. Two (3%) were diagnosed with overt adrenal CS. In addition, we included eight patients with pituitary CS. Pre- and postoperative samples were obtained from 13 patients, and 4 patients provided only postoperative samples. The postoperative specimens were collected a median of 24 (range 11–61) months after surgery. All surgically treated patients were biochemically cured at the time of sample collection and had been without corticosteroid supplements for at least 6 months.

The healthy controls did not have hypertension, hypercholesterolaemia, diabetes mellitus, and osteoporosis and used no medication. The metabolic complications among the patients are given in Table 1. Fourteen patients (19%) had two or more of these comorbidities. Eight patients (11%) were previously diagnosed with cardiovascular disease, and five (6.8%) had previous cerebrovascular stroke. There was no difference in systolic and diastolic blood pressures between patients and control subjects at the time when blood samples were drawn.

Inflammatory biomarkers

Forty-nine of the 92 inflammatory and cardiovascular biomarkers differed significantly between patients with ACS/CS and healthy controls, after correction for possible FDR and smoking habits (for complete list, see Table S2). Forty-six biomarkers were upregulated in patients, and three were downregulated (Figures 1 and 2). In the healthy control group, three of the biomarkers had one significant outlier, and these biomarkers were excluded (see Table S1).

Variation of biomarkers according to degree of hypercortisolism

None of the 49 biomarkers differed between patients with overt CS and ACS and not between ACS and possible ACS.

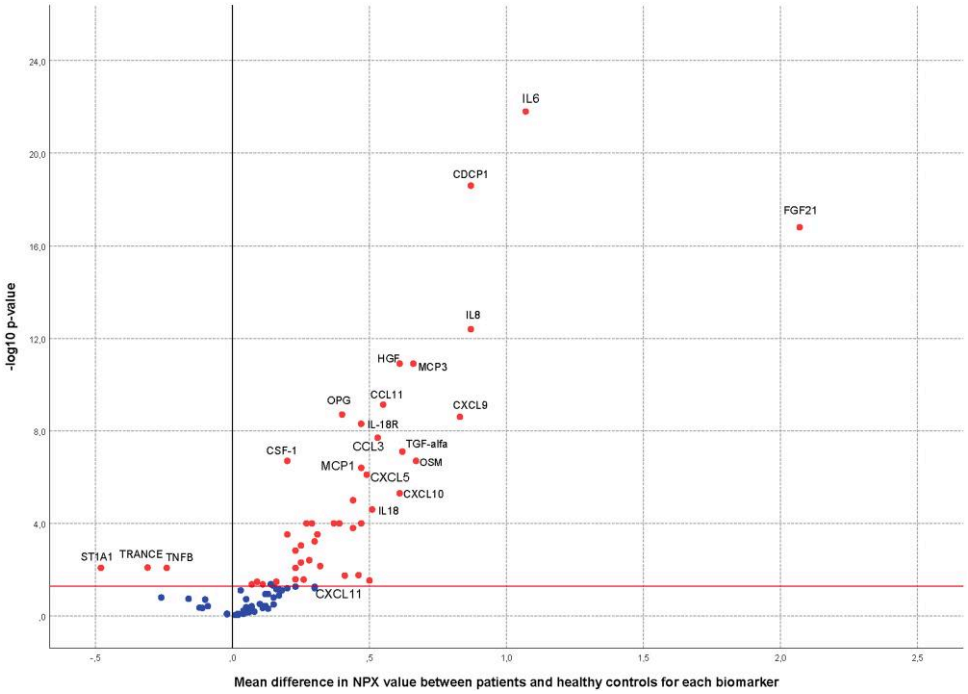

Figure 1. Volcanoplot showing significantly (red) and non-significantly (blue) higher and lower levels of biomarkers in patients with ACS/CS compared with healthy controls, respectively.

Oncostatin-C (OSM) and transforming growth factor alpha (TGFα) showed positive correlation with morning serum cortisol levels in the ACS/CS patients, but none of them correlated positively with post-DST cortisol level or 24 h UFC level. Fibroblast growth factor 21 (FGF-21) was significantly higher in patients with diabetes (median 7.2, range 4.6–10.6) compared with non-diabetic patients (median 6.4, range 2.6–11.9). For hypertension, dyslipidaemia, and osteoporosis, there were no significant differences between biomarker levels in patients with and without the particular metabolic manifestation.

Preoperative contra postoperative biomarker levels

From seventeen surgically cured patients (ACS or CS), postoperative samples were collected between 11 and 61 months after operation (median time 24 months) and at least 6 months after tapered off cortisone treatment. All patients were then biochemically cured. In these patients, 5 out of 49 significantly different biomarkers showed some degree of normalization (interleukin 33 [Il33], OSM, tumour necrosis factor receptor superfamily member 9 (TNFRSF9), C-X-C motif chemokine 11 (CXCL11), and interleukin-15 receptor subunit alpha [Il-15RA]), but the changes were not significant after correction for multiple analyses. Wilcoxon signed rank test did not show significantly differences pre- and postoperatively for any of the biomarkers ($P > .05$). Figure 3 shows the differences in biomarker levels between healthy controls and patients pre- and postoperatively, respectively. The biomarkers are grouped, and the NPX values are summed according to which metabolic complication they are mainly associated with.

Correlation of biomarkers with age, sex, BMI, smoking habits, and kidney function

In our assessment of biomarkers in the ACS/CS group, we used reference ranges based on the healthy control group data (2.5% and 97.5% percentile). Correlation analyses for the 46 biomarkers were performed on the healthy control group to evaluate the need of sex-, BMI-, and age-specific reference ranges. Ten biomarkers correlated with age, of which five markers were positively associated and five were negatively associated (Table S2). Regression analyses with adjustment for age did not affect our findings, further supported by no difference in median levels between age groups of healthy controls for the relevant biomarkers. This indicates that age cannot explain the increased levels in patients compared with healthy controls.

Ten of 46 biomarkers showed sex differences. Five were higher in women, and this could potentially impact the assessment of these biomarkers in the ACS/CS group as it consisted of proportionally more women. However, when comparing these five biomarkers only in female participants (patients against healthy controls), we found that they remained significantly higher in the ASC/CS group. Five biomarkers were higher in men (Table S2).

Four of 46 biomarkers correlated with BMI, but only 1 showed a positive association (interleukin-6 [IL6]). After linear regression with correction for BMI, there was still a significant difference in IL6 levels between the patients and healthy controls ($P < .05$). Three biomarkers correlated negatively with BMI (Table S2).

No difference was found in median levels between smokers and non-smokers in healthy controls for the significantly different biomarkers. Linear regression was also used to correct for smoking habits, without affecting the differences found between healthy controls and diseased patients.

All but one of the patients had estimated glomerular filtration rate (eGFR) ≥ 60 mL/min/1.73 m^2, corresponding to chronic kidney disease (CKD) stage 1 or 2. The last one had CKD stage 3b. There were no significant differences in biomarker levels between patients among CKD categories.

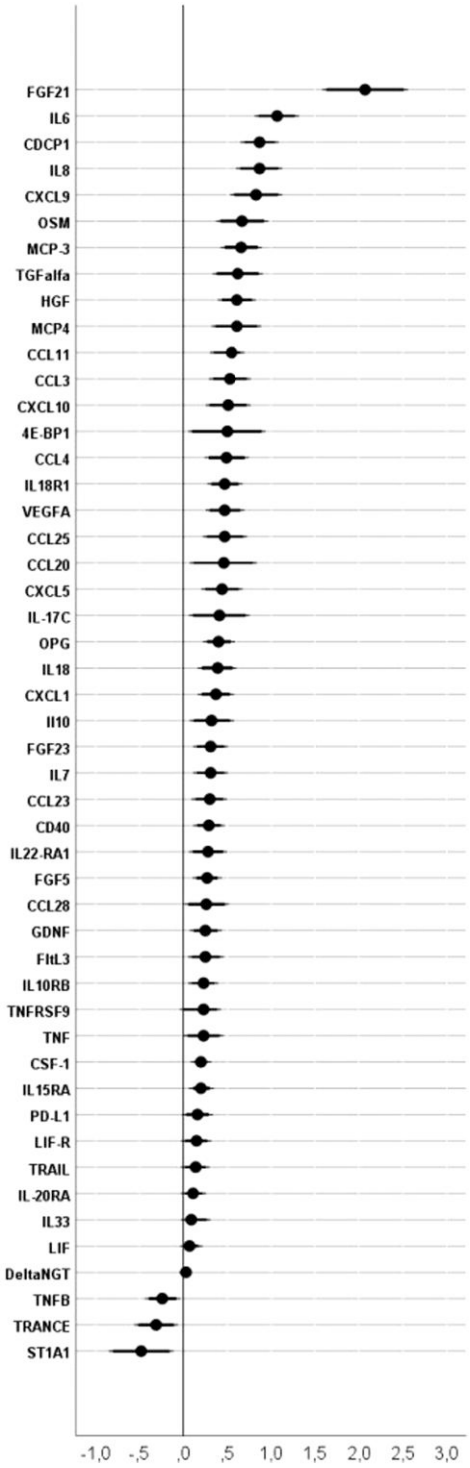

Figure 2. Forest plot showing the difference in NPX means (with confidence intervals) between patients and healthy controls for the significantly different biomarker.

The three biomarkers that showed significantly lower levels in the ACS/CS group compared with healthy controls were TNF-related activation-induced cytokine (TRANCE), tumour necrosis factor-β (TNFβ), and sulfotransferase 1A1 (ST1A1). Significant differences in these biomarkers between the patients and healthy controls ($P < .05$) remained after linear regression with correction for age and BMI.

Reproducibility of biomarker levels

Four ACS patients had delivered two preoperative samples, with the same degree of cortisol excess at both time points. The time between each sample was 11, 12, 12, and 14 months, respectively. Except for biomarkers CXCL9 and IL-15RA, there were no significant differences between biomarker levels in the first and last samples, indicating excellent test reproducibility.

Discussion

Patients with ACS and overt CS have a broad increase in inflammatory biomarkers, several of which are related to cardiometabolic disease. The biochemical degree of hypercortisolism did not reflect the increase in biomarker levels, as the biomarker levels did not correlate with the post-DST cortisol levels, the 24 h UFC level, or the morning serum cortisol levels. There was no significant difference between biomarker levels in patients with ACS and overt CS. Surprisingly, biomarker levels continued to be elevated even after biochemical cure. This suggests that inflammatory changes are imprinted and that the patients could carry an increased risk of cardiovascular events and metabolic complications even after successful treatment of hypercortisolism.

A few previous studies have shown an increase in one or a few protein markers in CS, but a more complete serum proteome in CS and ACS is still to be defined. Specifically, proinflammatory cytokines, such as IL6, interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α), have been demonstrated at increased systemic levels in patients with CS compared with BMI-matched healthy controls,[26-28] in line with our data. Moreover, the elevated proinflammatory cytokines appear persistent over 1 year after surgical remission, despite improvements in body composition and insulin sensitivity.[29] These findings are supported by our study showing a lack of normalization after successful surgical treatment. This state of chronic low-grade inflammation may be a major contributor to the increased cardiovascular morbidity and mortality found in patients with ACS and CS, which persists even after surgical remission.[36,37] Unexpectedly, the association of inflammatory markers with cardiometabolic risk factors (except for type 2 diabetes and FGF-21) was not specifically observed in this study. A possible explanation might be the small sample size, giving low number of patients with hypertension, diabetes, dyslipidaemia, and osteoporosis. Even though we did not find correlation between 24 h UFC and inflammation, a previous paper by Ceccato et al.[38] found 24 h UFC to correlate with cardiovascular events. Furthermore, they found increased amount of visceral adipose tissue in CS patients contributing to increased cardiovascular risk.[39]

Furthermore, IL6 is a non-specific marker of inflammation and associated with risk of cardiovascular events.[40] The increased IL6 levels in our patients may reflect an altered balance between proinflammatory and anti-inflammatory cytokines, inducing a state of chronic inflammation.

FGF-21 is a cytokine that regulates glucose and lipid metabolism, with increased levels being reported in type 2 diabetes.[41-43] In our study, FGF-21 was higher in ACS/CS patients compared with healthy controls and even more so in patients that had type 2 diabetes. Interestingly, ACS/CS patients without known type 2 diabetes also showed higher levels than healthy controls. FGF-21 has been identified as a promising treatment target for metabolic disease like type 2

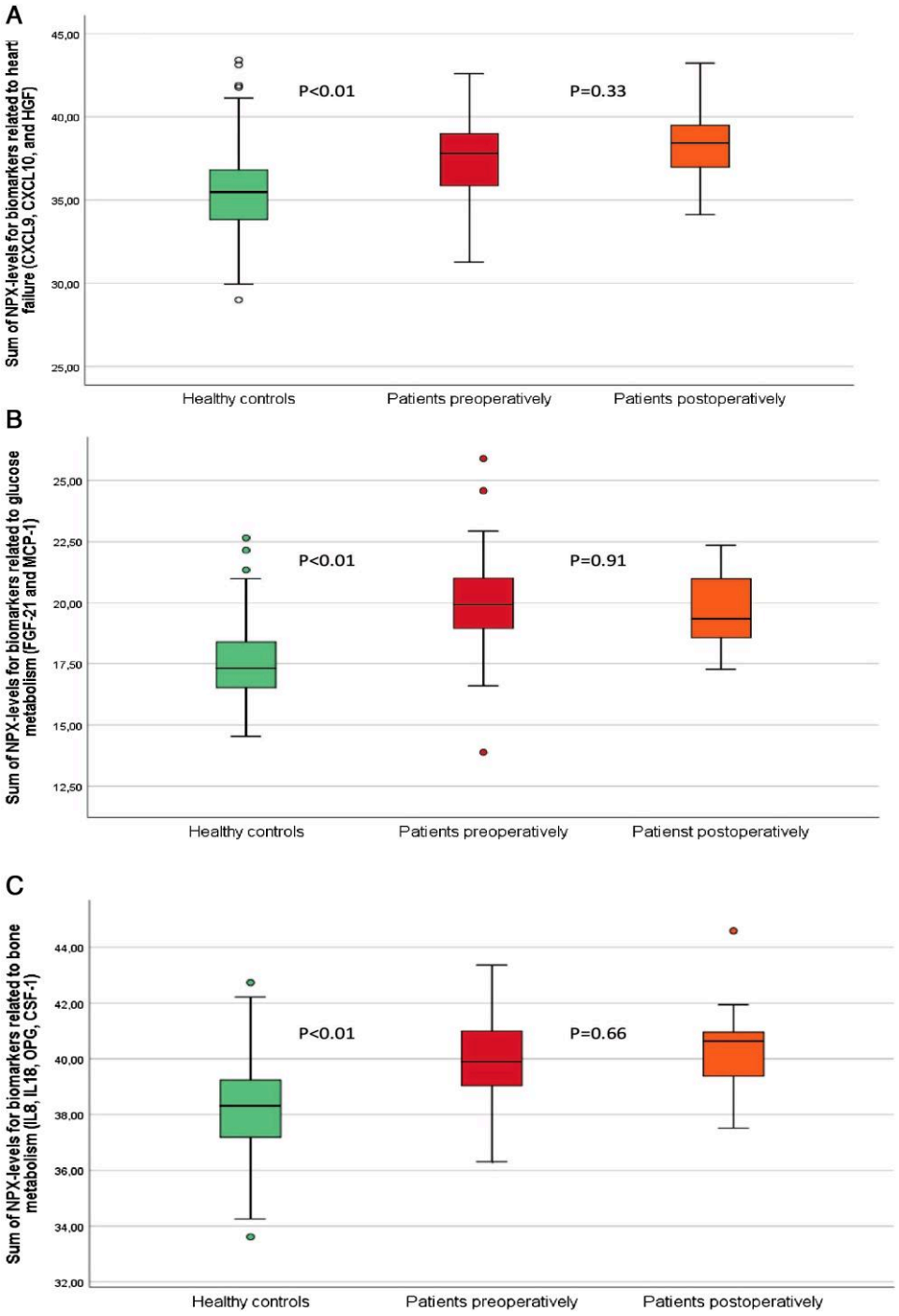

Figure 3. Boxplot showing the distribution of biomarker levels related to heart failure (A), glucose metabolism (B), and bone metabolism (C), in healthy controls, compared with patients preoperatively and postoperatively, respectively. All panels show significantly higher levels in ACS/CS patients compared with healthy controls and a lack of normalization postoperatively. (The biomarkers are grouped and the NPX levels are summed according to which metabolic complication they are mainly associated with.) Sum of NPX values on the *y*-axis.

diabetes, obesity, and non-alcoholic steatohepatitis.[44] Monocyte chemotactic protein-1 (MCP-1) is another chemokine playing a key role in insulin resistance, diabetes, and its complications such as diabetic nephropathy and retinopathy. The level of circulating MCP-1 is significantly increased in patients with type 1 and type 2 diabetes.[45] MCP-1 is an adipokine whose increased expression by adipose tissue can induce insulin resistance and infiltration of macrophages in adipose tissue. We found increased levels of MCP-1 in the ACS/CS group whether they had type 2 diabetes or not.

Our data showed significantly increased levels of chemokine (C-X-C motif) ligand (CXCL) 9 and 10 in ACS/CS patients compared with healthy controls. To the best of our knowledge, these markers are not previously linked to hypercortisolism. These chemokines are valid biomarkers predicting the development of heart failure and left ventricular dysfunction and could be involved in the pathophysiology causing adverse cardiac remodelling.[46,47] Also, hepatocyte growth factor (HGF), which was significantly elevated in our ACS/CS patients, have been described to play a major role in cardiac

function and remodelling. The serum levels are strongly related to mortality in chronic systolic heart failure.[48]

Some biomarkers known to be involved in atherosclerosis are also elevated in our study, such as monocyte chemotactic protein-3 (MCP-3). The upregulation of MCP-3 has been associated with inflammatory states including infection, cardiovascular disease, and tumour microenvironment.[49] Moreover, CXCL5 and chemokine C-C ligand 23 (CCL23) expression is increased in patients with atherosclerosis regardless of known preexisting cardiovascular disease,[50] and these biomarkers were elevated in our ACS/CS cohort.

Several studies have shown that bone mass as well as bone area is reduced in endogenous CS, likely due to decreased bone formation and increased bone resorption.[51-53] A Norwegian study from Kristo et al.[54] found an increased level of the proinflammatory cytokines IL8 and IL18 in patients with CS compared with healthy controls. These cytokines may be involved in the pathogenesis of disturbed bone homeostasis in CS. Also, Camozzi et al. showed that patients with CS have increased serum osteoprotegrin (OPG) levels that remain unchanged after recovery, despite a restoration of bone formation. The authors correlated the absence of OPG normalization with a persistent increased inflammation pattern, which might represent a pro-atherogenic profile[55] reflecting an ongoing damage of the glucocorticoids on the cardiovascular system. Elevated colony-stimulating factor 1 (CSF-1) supports this, as CSF binds to receptors on osteoclasts and ultimately leads to increased plasma calcium levels, through the resorption of bone. This is in coherence with our proteomic study as a systematic elevation of IL8, IL18, OPG, and CSF-1, indicating a negative impact of bone metabolism in ACS and CS.

A similar large-scale mapping of biomarkers using the same analysis method was previously done in autoimmune Addison disease, finding that 17 out of 92 studied inflammatory markers differed significantly between patients with adrenal insufficiency and healthy controls.[56] The authors concluded that the chronic replacement of glucocorticoids had an unfavourable effect on proinflammatory and cardiovascular markers, which could explain the increased risk of cardiovascular disease in these patients. The panel they used is only partly overlapping with our panel, and IL6 was found elevated in Addison patients just like we found in our ACS/CS patients. Osteoprotegrin on the other hand was significantly lower in Addison patients compared with healthy controls, in contrast to our ACS/CS patients that had significantly elevated levels also for OPG.

Previous data have shown increased inflammation with age,[57] BMI,[58] hypertension,[59] and diabetes.[60] Except for FGF-21 being higher in patients with diabetes compared with non-diabetic patients, we found no other significant correlations or differences between biomarker levels in patients regardless of age, BMI, or presence of hypertension, dyslipidaemia, or diabetes.

There are some limitations to our study. First, smoking habits were different in healthy controls and patients. This could be a major confounder, as smoking is a known modulator of the immune system. We used linear regression to adjust for smoking habits and found that smoking did not affect our findings. Second, in determining the broad panel of 92 biomarkers, there is a risk of chance findings. However, the vast number of proteins found to be different between the ACS/CS and healthy control group indicates that our findings are overall robust. We also applied appropriate statistical procedures to adjust for multiple testing. Furthermore, the healthy subjects were not screened with CT scan or DST to exclude adrenal incidentalomas and/or cortisol excess. In addition, the group of overt CS is small. This group was not the focus of this study paper but included to present a broad range of hypercortisolism. In addition, the operated group is small, and the findings must be interpreted with caution. Finally, the duration of exposure to cortisol excess and the age of debut which could be important factors in determining metabolic and cardiovascular changes could not be determined.

In conclusion, glucocorticoids act on nuclear receptors and regulate the expression of more than 3000 genes. What pathways are activated depends on the degree of hypercortisolism and the pulsatility of the cortisol secretion.[61] We found that patients with hypercortisolism, both overt CS and low graded ACS, have a systemic rise in inflammatory biomarkers involved in cardiometabolic processes, indicating an increased risk of obesity, insulin resistance, dyslipidaemia, and cardiovascular disease. However, this inflammatory burst was unaffected by the degree of hypercortisolism and its treatment, which may indicate an inflammatory imprinting and persistent risk of cardiovascular disease also after cure of hypercortisolism.

Supplementary material

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

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Conflict of interest: The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Data availability

All data sets generated during and/or analysed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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