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Clinical Research Article

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Potential Transcriptional Biomarkers to Guide Glucocorticoid Replacement in Autoimmune Addison's Disease

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Abbreviations: AAD, autoimmune Addison's disease; ACTH, adrenocorticotropic hormone; CA, cortisone acetate; CSHI, continuous subcutaneous hydrocortisone infusion; CV, coefficient of variation; FDR, false discovery rate; GC, glucocorticoid; HC, hydrocortisone; OHC, oral hydrocortisone replacement; rt-PCR, real-time polymerase chain reaction.

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Abstract

Background: No reliable biomarkers exist to guide glucocorticoid (GC) replacement treatment in autoimmune Addison's disease (AAD), leading to overtreatment with alarming and persistent side effects or undertreatment, which could be fatal.

Objective: To explore changes in gene expression following different GC replacement doses as a means of identifying candidate transcriptional biomarkers to guide GC replacement in AAD.

Methods: Step 1: Global microarray expression analysis on RNA from whole blood before and after intravenous infusion of 100 mg hydrocortisone (HC) in 10 patients with AAD. In 3 of the most highly upregulated genes, we performed real-time PCR (rt-PCR) to compare gene expression levels before and 3, 4, and 6 hours after the HC infusion. Step

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2: Rt-PCR to compare expression levels of 93 GC-regulated genes in normal versus very low morning cortisol levels in 27 patients with AAD.

Results: Step 1: Two hours after infusion of 100 mg HC, there was a marked increase in *FKBP5, MMP9,* and *DSIPI* expression levels. *MMP9* and *DSIPI* expression levels correlated with serum cortisol. Step 2: Expression levels of *CEBPB, DDIT4, FKBP5, DSIPI*, and *VDR* were increased and levels of *ADARB1, ARIDB5,* and *POU2F1* decreased in normal versus very low morning cortisol. Normal serum cortisol levels positively correlated with *DSIPI*, *DDIT4,* and *FKBP5* expression.

Conclusions: We introduce gene expression as a novel approach to guide GC replacement in AAD. We suggest that gene expression of *DSIPI, DDIT4,* and *FKBP5* are particularly promising candidate biomarkers of GC replacement, followed by *MMP9, CEBPB, VDR, ADARB1, ARID5B*, and *POU2F1*.

Key Words: Addison's disease, primary adrenal insufficiency, biomarkers, gene expression, glucocorticoid

In autoimmune Addison's disease (AAD), patients suffer from deficiency of glucocorticoids (GCs) and mineralocorticoids due to autoimmune destruction of the adrenal cortex, a fatal condition if left untreated [1]. Current treatment strategies rely on replacement of cortisol (the main GC) and aldosterone (the main mineralocorticoid), usually twice or thrice oral hydrocortisone (HC) or cortisone acetate in combination with once daily oral fludrocortisone and salt. Conventional treatment strategies fail to restore good health in patients with AAD, evident by lower quality of life [2, 3], increased prevalence of cardiovascular disease, metabolic syndrome, and infections as well as overall higher mortality rates [4-7].

In addition to the lethal threat of acute adrenal crisis, current evidence points to 2 main causes of the deleterious health outcomes in AAD. First, none of the currently used treatment modalities perfectly mimic the physiological circadian rhythm of GC production. Second, there is no biomarker available to aid physicians in determining the correct GC replacement dosage for each individual [8, 9]. Instead, GC dosages are adjusted based on the patient's symptoms, signs, and general well-being. Overtreatment may be especially challenging to identify, as clinical clues of too-high GC replacement are nonspecific and slow to develop, including weight gain, metabolic syndrome, hypertension, and sleep disturbances [10].

Despite numerous attempts, measurement of GCs and adrenocorticotropic hormone (ACTH) in blood, urine, and saliva fall short to guide GC replacement [11]. In the last decade, hair cortisol concentration has emerged as a promising tool for assessing GC exposure over time [9]. On the downside, this approach requires a relatively large hair sample (1 cm thick), and is therefore not suited for frequent assessment, and in bald patients, not at all. Although measurement of GC levels in body fluids or tissue will provide information on the bioavailability of the exogenous GC in AAD, it does not reflect the actual physiological effect [12].

Gene expression is an alternative avenue that allows for evaluation of GC effects at a transcriptional level in the individual patient. Through binding to the GC receptor, GCs regulate the expression of several hundred genes involved in vital physiological processes, including metabolic hemostasis, stress response, and immunity [13]. Previous studies suggest that expression levels of GC-regulated genes align with the reported use and dosage of exogenous GC [14]. Yet, its potential use for guiding GC treatment in AAD remains an unexplored landscape.

We conceived this study in an aspiration to improve health and quality of life in patients with AAD by personalizing GC replacement dosages. In order to foresee positive and adverse effects of different GC dosages, we need access to biomarkers that can act as sensors of GC treatment. Here, we set out to identify candidate GC biomarkers among GC-responsive genes, characterized by change in gene expression levels as a response to different levels of GC exposure.

Patients and Methods

Study Design

In a two-step approach, we investigated the expression of GC-regulated genes in different settings of GC replacement in patients with AAD: first, after a high-dose stress test with intravenous 100 mg HC, and second, at normal morning cortisol compared to very low morning cortisol levels. In the second step, normal morning cortisol was achieved through near-physiological replacement with continuous subcutaneous hydrocortisone infusion (CSHI) and very low morning cortisol levels through an over-night (> 15 hour) fast from conventional oral hydrocortisone replacement treatment (OHC).

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In the first step, we wanted to explore the immediate effects of high-dose HC exposure on 3 selected genes. In the second step, we compared expression levels of a selected large range of GC-regulated genes in patients with AAD with normal morning cortisol versus very low morning cortisol levels.

Step 1: Gene Expression in Response to 100 mg Hydrocortisone

Patients

Ten patients (50% females) with verified AAD were recruited from the National Registry for Addison's disease (ROAS). Their median daily cortisone acetate dose was 37.5 mg/day (12.5-50 mg/day), equivalent to 30 mg/day HC (10-40 mg/day). Blood samples were collected at 8 to 9 AM (time point 0), after having abstained from cortisone acetate treatment for 18 hours, and repeated at 2 (2h), 4 (4h), and 6 (6h) hours after intravenous infusion of 100 mg HC. Samples were anonymized. All subjects provided written informed consent and ethical permission was granted prior to study start (Norway REK Vest no. 014.03).

RNA purification from blood

Whole blood was sampled into Tempus Blood RNA tubes at time points 0, 2h, 4h, and 6h, and RNA extraction from leukocytes performed on the 6100 Nucleic Acid PrepStation (Applied Biosystems, USA). Amount and quality of the extracted RNA was verified by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). All RNA samples were qualitatively adequate with RNA integrity numbers between 6.9 and 9.7.

Global transcriptional analysis in blood using RNA expression microarray

Initially, RNA samples from time points 0 and 2h from all 10 patients were included. Microarray experiments were performed using the Applied Biosystems 1700 Expression Array system. An amount of 800 ng of total RNA from each sample was reverse transcribed, amplified, and DIG-labeled (DIG-dUTP; Roche, Germany), using the Applied Biosystems NanoAmp RT-IVT Labeling Kit. The amount (82-147 µg) and the quality of the DIG-labeled cRNA was controlled by both NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer. All samples except one passed the qualitative analysis, and both samples from this individual were removed. Hence, we proceeded with 18 samples. Then, 20 µg of DIG-labeled cRNA was hybridized to the Applied Biosystems Human Genome Survey Microarray v.1.0. The chemiluminescent signal detection, image acquisition, and image analysis of the microarrays were performed on the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer. In addition to the QC report generated by the AB1700 scanner software, the integrated R software was used to control data integrity graphical outputs. Data was loaded onto BioArray Software Enviroment (BASE), and PANTHER was used to annotate the probe IDs.

Among the 150 most upregulated genes in the global transcription analysis, we selected 3 for further investigation (Supplementary appendix 1 [15]): FK506 binding protein 51 (*FKBP5*), matrix metallopeptidase 9 (*MMP9*), and delta sleep inducing peptide immunoreactor (*DSIPI*; also known as *TSC22D3* and *GILZ)*. *FKBP5* was selected as it is an important short-loop feedback inhibitor of GC action [16]; *MMP9* because mounting evidence points at its potential as a biomarker in multiple conditions, including cardiovascular [17, 18] and inflammatory diseases [19, 20]; and *DSIPI* for being a multi-tissue biomarker of GC action [21].

Real-time PCR verification of differentially expressed genes following 100 mg HC intravenously

Real-time PCR (rt-PCR) verification of the microarray-data at time points 0, 2h, 4h, and 6h was done for *FKBP5, MMP9,* and *DSIPI* using the High-Capacity RNA-to-cDNA Kit and commercially available Taqman-probes. Glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) was applied as housekeeping gene. The PCR amplification protocol was denaturation at 95 °C for 10 minutes, followed by 40 amplification cycles each at 95 °C in 15 seconds and 60 °C for 1 minute. All samples were run in triplicates on the 7900HT PCR system. The ΔΔCt method was applied to calculate differences between the time points [22].

Hormone analysis

Venous blood was collected at time points 0 and 2h for analysis of serum cortisol and plasma ACTH. The hormone analyses were done using immunoassay kits from Diagnostic Products Corp (Los Angeles, CA, USA; Siemens, Cat# L5KCO2, RRID:AB_2877715, and Siemens, Cat# L5KAC2, RRID:AB_2877714) at Haukeland University Hospital, Bergen, Norway, both assays with a coefficient of variation (CV%) <10%.

Step 2: Gene Expression in Normal Versus Very Low Morning Cortisol Levels

Patients and treatment modalities

Patient selection and study design has been described in detail elsewhere [23]. In short, a prospective, randomized, cross-over study was conducted, comparing 12 weeks

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treatment with OHC versus CSHI. The 2 treatment periods were separated by a minimum 8 weeks washout, in which the patient followed his or her usual GC replacement. At the end of each of the 12-week treatment period, the patients returned to the hospital for blood sampling at 8 AM.

For the present study, we used the 2 treatment modalities as a means of controlling morning GC exposure. When treated with OHC, patients were asked to take their final HC dose before 5 PM the day before testing and were practically cortisol depleted upon testing. When treated with CSHI, however, patients continued to receive HC infusions, meaning the blood samples were collected shortly after the simulated morning cortisol peak. For the rest of this paper, we therefore refer to the 2 different GC replacement situations as *very low cortisol* (>15 hour fasting from OHC) and *normal cortisol* (CSHI).

Our study cohort consisted of 27 patients with AAD, among whom 18 patients were from Norway and 9 from Sweden (Table 1). The original study cohort included 5 additional patients who were not included here due to lack of RNA samples. All participants provided written informed consent, and ethical approval was granted in both countries before study start (EudraCT #2009-010917-61; NCT 01063569).

Transcriptional and hormonal analysis in blood

All patients provided whole blood, serum, and plasma samples before and after 12 weeks in each treatment arm. Serum cortisol was analyzed by liquid chromatography mass spectrometry [24] and plasma ACTH by chemiluminescent immunometric assay (Immulite 2000; Siemens AG, Munich, Germany; Siemens Cat# L2KAC2, RRID:AB_2783635) with a CV% of ≤10.1% and ≤8%, respectively.

RNA extraction from leukocytes was done using the Tempus Spin RNA Isolation Kit (Applied Biosystems). The cDNA was made using the RT2 First Strand Kit (Qiagen). A customized version of the RT^2 Profiler PCR Array Human Glucocorticoid Signaling kit (Qiagen) was then used in order to profile expression of genes of relevance to GC activity. Two of the controls in the panel occurred in triplets, and we replaced the 4 spare copies with *SPP1, ARNTL, ARNTL2,* and *MMP9* (Version 1). In a pilot analysis of version 1, we found 6 genes with gene expression levels below the threshold limit; these were replaced by genes previously shown to be significantly altered in patients with adrenal insufficiency (Version 2; *ADM, MMP12, CASP8, EN-RAGE, RETN, CXCL1*) [5]. The complete overview of the 93 genes included in Version 2 is listed in the Supplementary Appendix (S2) [15].

We analyzed the gene expression levels in 2 samples from each of the 27 patients, collected at the end of each 12-week treatment period. The assays were run according to the protocol of the manufacturer on the 7900HT PCR system (Applied Biosystems). Data was analyzed with the ΔΔCt method using the mean of 4 housekeeping genes as reference (*GADPH, HPRT1, RPLP0,* and *B2M*) [22].

Statistics

Descriptive data are presented as median with range. In step 1, normalized signal levels of each probe from the global transcription analysis were log2 transformed and then quartile normalized (Limma package, R software). To account for any negative intensity values produced by the normalization steps, all values had the lowest negative value added to make all intensity values zero or greater, which would then allow for fold change calculations to be carried out. A positive Log2 fold change value represents an increase in fold change where a value of 1 is equal to a doubling of the original value. Next, we performed *t* tests analysis along with a false discovery rate (FDR) adjustment. The 150 most upregulated genes were ranked according to log2 fold change after excluding genes with FDR above 0.01. Fold change for rt-PCR results was calculated as the ratio between the final over the initial gene expression level. We performed Spearman's correlation (IBM SPSS Statistics) for the relationship between hormone and gene expression levels. Statistical significance was set to 0.05. In step 2, we employed the Wilcoxon signed rank test (IBM SPSS Statistics) to compare normalized gene expression values in normal versus very low morning cortisol, presented as z-score where a score closer to 0 suggests even distribution between the groups. The effect size was calculated by dividing the z-score by the square root of the number of observations (ie, 2 observations for each case, N = 54). To minimize the risk of type 1 error, the significance value was set to $P < 0.01$. For calculation of fold change, the normalized gene expression value at normal serum cortisol was divided by the very low cortisol for each patient. In both steps, plasma ACTH levels exceeding the upper reference limit (278 pmol/L) were plotted as 278 pmol/L and the lower reference limit (1.1 pmol/L) was plotted as 1.1 pmol/L.

Table 1. Step 2: Baseline Patient Characteristics

Female, n (%)	20 (74%)
Age, years (range)	46 (20-66)
HC-eqv dosage, mg/ day (range)	25 (15-66)
BMI, kg/m^2 (range)	24.8 (18.3-37.4)

Abbreviations: BMI, body mass index; HC-eqv dosage; hydrocortisone equivalent dosage.

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Results

Step 1: Gene Expression in Response to 100 mg Hydrocortisone

Infusion of 100 mg HC to medication-fasting AAD patients had a profound short-term effect on the expression *FKBP5*, *MMP9*, and *DSIPI*; increased gene expression levels were evident after 2h and had further increased at 4h. At 6h, the expression levels stabilized at an upregulated level compared to before the HC infusion (Fig. 1). Log2 fold change was 0.429 ($P < 0.001$, FDR < 0.001) for *FKBP5*, 0.560 ($P < 0.001$, FDR < 0.002) for *MMP9*, and 0.274 ($P < 0.001$, FDR < 0.002) for *DSIPI* (S1).

Individual serum cortisol and plasma ACTH levels are listed in Table 2. At 2h, there was a strong positive correlation between serum cortisol levels and the gene expression levels of *DSIPI* ($r = 0.709$, $P < 0.022$) and *MMP9* ($r = 0.673$, $P < 0.033$), but not for *FKBP5* ($r = 0.442$, $P < 0.200$). No significant associations were found between ACTH and gene expression levels (data not shown).

Step 2: Gene Expression in Normal Versus Very Low Morning Cortisol Levels

Eight of the 93 investigated genes were significantly different in normal compared to very low cortisol levels (Fig. 2). *DDIT4* (z = –3.05, $P < 0.002$), *CEBPB* (z = –2.84, $P < 0.005$), *DSIPI* (z = –2.82, $P < 0.007$), *FKBP5* (z = –2.79, $P < 0.005$), and *VDR* (z = –2.67, $P < 0.008$) revealed increased expression, while *ADARB1* (z = –3.84, $P < 0.001$), *POU2F1* (z = –2.79, $P < 0.005$), and *ARID5B* (z = –2.64, $P < 0.008$) were decreased. The effect size was largest for *ADARB1* ($r = 0.52$), followed by *DDIT4* ($r = 0.42$), *CEBPB* ($r = 0.39$), *FKBP5* ($r = 0.38$), *POU2F1* ($r = 0.38$), *DSIPI* ($r = 0.37$), *VDR* ($r = 0.36$), and *ARID5B* ($r = 0.36$). Fold change was greatest for *DDIT4* (md = 2.32 [0.34, 10.00]) and *FKBP5* (md = 2.03 [0.43, 6.28]) (Fig. 3).

As expected, median serum cortisol was significantly higher and plasma ACTH significantly lower at normal cortisol exposure compared with very low cortisol (cortisol; 277 nmol/L [7, 467] vs 3 nmol/L [0.2, 32], $P < 0.0001$, ACTH; 7.3 pmol/L [1.1, 276] vs. 123 pmol/L [1.1, 278], $P < 0.0001$, respectively). At normal cortisol levels, serum cortisol correlated negatively with plasma ACTH ($r = –0.435$, $P < 0.030$) and positively with *DDIT4*, *FKBP5*, and *DSIPI* ($r = 0.450$, $P < 0.021$; $r = 0.419$, $P < 0.033$; and $r = 0.400$, $P < 0.043$, respectively). At very low cortisol levels, no correlations were found for serum cortisol and gene expression levels, but plasma ACTH negatively correlated with gene expression levels of *DDIT4* and *FKBP5* ($r = –0.411$, $P < 0.033$ and $r = –0.399$, $P < 0.039$, respectively).

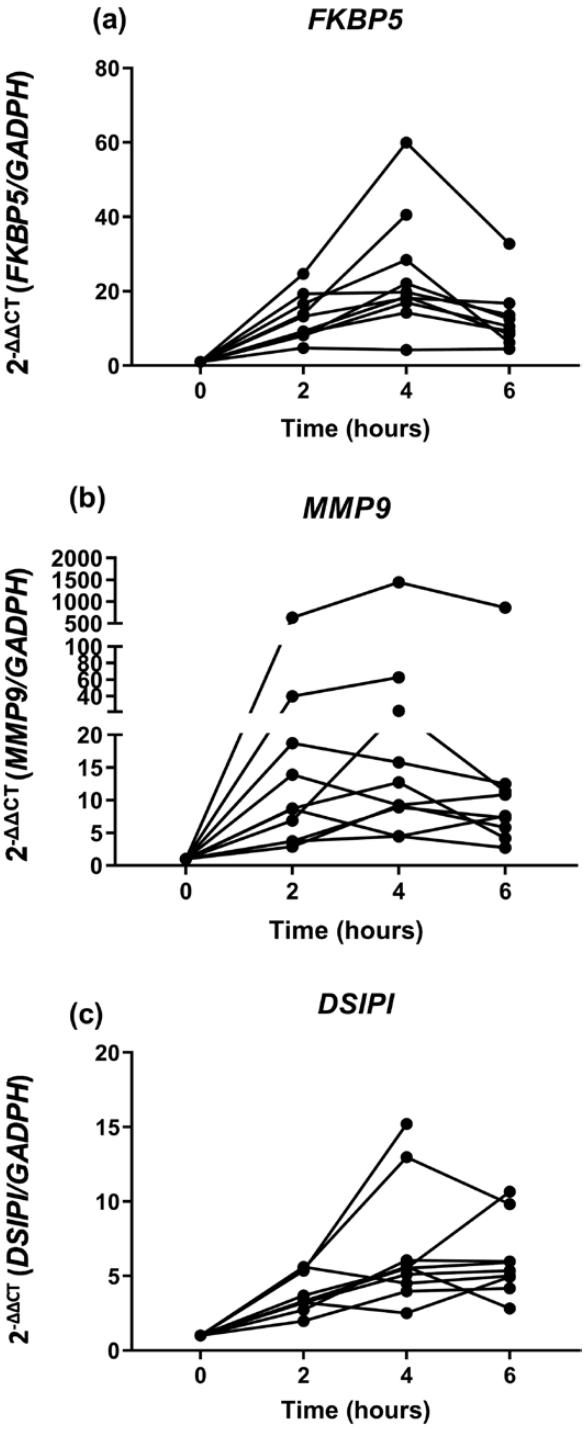

Figure 1. Gene expression of *FKBP5* (a), *MMP9* (b), and *DSIPI* (c) before and up to 6 hours after intravenous infusion of 100 mg hydrocortisone.

Expression levels of *DSIPI*, *FKBP5*, and *DDIT4* strongly correlated with each other at both normal and very low cortisol levels (*DSIPI* and *FKBP5:* $r = 0.705$, $P < 0.001$ and $r = 0.706$, $P < 0.001$; *DSIPI* and *DDIT4:* $r = 0.695$, $P < 0.001$ and $r = 0.702$, $P < 0.001$; *FKBP5* and *DDIT4*: $r = 0.573$, $P < 0.001$ and $r = 0.692$ $P < 0.002$, respectively). Other significant correlations were found between

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Table 2. Step 1: Serum Cortisol and Plasma ACTH Before and 2 Hours After Intravenous Infusion of 100 mg Hydrocortisone in Patients With AAD.

Patient	Before hydrocortisone		2 hours after hydrocortisone	
	Serum cortisol, nmol/L	Plasma ACTH, pmol/L	Serum cortisol, nmol/L	Plasma ACTH, pmol/L
1	<28	>280	1879	>280
2	<28	>280	1490	104.6
3	<28	>278	1862	24.2
4	<28	>278	1672	35.7
5	<28	39.1	2621	3.5
6	<28	15.3	2687	4.1
7	<28	178	1333	11.4
8	109	198	1708	15.1
9	<28	<1.1	2759	5.5
10	<28	<1.1	1873	<1.1

Abbreviations: AAD, autoimmune Addison's disease; ACTH, adrenocorticotropic hormone.

CEBPB and *DSIPI* ($r = 0.564$, $P < 0.001$ and $r = 0.584$, $P < 0.002$) and *CEBPB* and *FKBP5* ($r = 0.522$, $P < 0.005$ and $r = 0.521$, $P < 0.005$) at normal and very low cortisol levels, respectively.

Discussion

Biomarkers reflecting GC replacement in AAD are lacking. In this exploratory study, we mapped the transcriptional landscape of GC exposure and found that *DSIPI, DDIT4,* and *FKBP5—independently* and as a triad—are candidate transcriptional biomarkers reflecting levels of GC because (i) all 3 genes were clearly and consistently upregulated after both high-dose HC infusion and in normal cortisol compared to very low cortisol, (ii) gene expression levels significantly correlated with normal cortisol levels, and (iii) they strongly correlated with each other, implying a high degree of co-regulation.

DSIPI, a key mediator of GC anti-inflammatory effects [25], appears as the most precise biomarker since its expression significantly correlated with serum cortisol both after high-dose HC infusion (step 1) and with normal cortisol levels (step 2). *DDIT4,* an important inhibitor of the mTOR pathway in response to stress [26], and *FKBP5,* an important short-loop feedback inhibitor of GC action [16], may be more sensitive markers of GC exposure as they revealed the largest fold changes between very low and normal cortisol levels (step 2). Finally, *DDIT4* and *FKBP5* were the only genes where expression levels significantly correlated with plasma ACTH at very low cortisol.

In addition to *DSIPI* and *FKBP5,* we found that that *MMP9* had a rapid and sustained increase in gene expression following infusion of 100 mg HC (step 1). This is in contrast to a study by Aljada and coworkers where plasma MMP9 protein levels significantly decreased after 100 mg HC infusion [27], but in line with another study on healthy individuals where MMP9 levels increased in response to 300 mg HC infusion [28]. Obviously, gene expression and plasma protein levels are not directly comparable, but we would expect the direction of the response to HC infusion to correspond. Taken together, this indicates that more research is needed to determine the true effect of high-dose HC exposure on *MMP9* gene expression and MMP9 protein levels.

In step 2, 5 of the 93 GC-regulated genes were significantly upregulated (*DDIT4, DSIPI, FKBP5*, *CEBPB, VDR*) whereas 3 were downregulated *(ADARB1, POU2F1, ARID5B*) in normal compared with very low cortisol. Increased *CEBPB* expression has been suggested as an early marker of efficacious response to GC treatment in inflammatory bowel disease (IBD) and could be used to identify the 20% of IBD patients who are refractory to GC treatment [29]. With this in mind, we suggest that future studies explore whether difference in *CEBPB* expression could help identify patients with AAD in need of higher GC replacement dosages due to partial GC resistance.

Expression of *VDR* has previously been shown to increase following GC exposure in a time- and dose-dependent manner, but only in the presence of GC receptor, indicating that GCs directly regulate *VDR* expression through GC receptor activation [30].

The largest effect size on gene expression change was noted for *ADARB1,* a gene considered key for circadian rhythmicity [31]. Previous studies disagree on whether GCs increase or decrease *ADARB1* expression. In an in vitro study on subcutaneous preadipocytes, expression of ADARB1 was twice as high in cells treated with cortisol compared with untreated cell controls [32]. In contrast,

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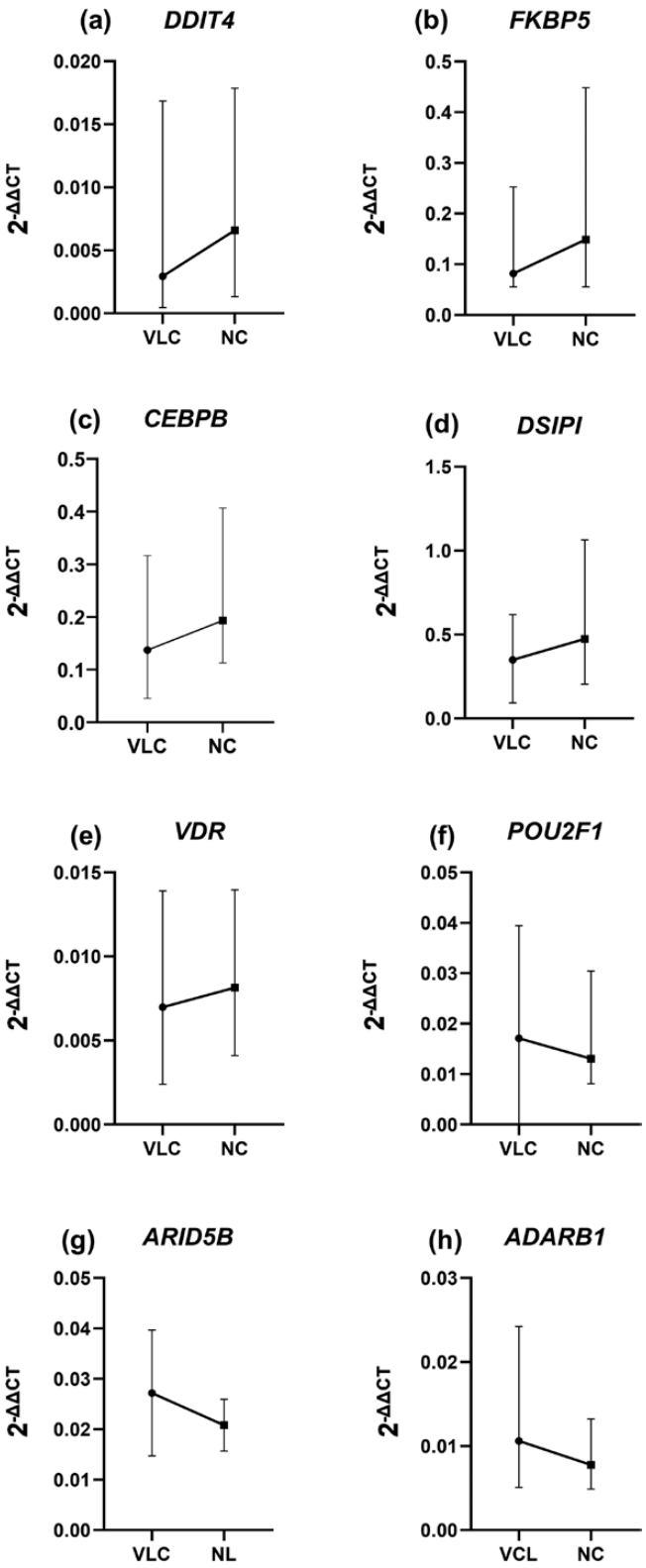

Figure 2. Genes with significant change in expression levels in very low cortisol (VLC) compared with normal cortisol (NC) levels. The black circles and black squares mark the median values for VLC and NC, respectively, and the whiskers mark the range.

our data adds evidence to the notion that GCs suppress *ADARB1* expression, as previously demonstrated in human keratinocytes treated with dexamethasone [33].

For *POU2F1*, a transcription factor important in stem cell regulation, our findings support that GCs suppress its expression in line with previous studies [34]. The clinical

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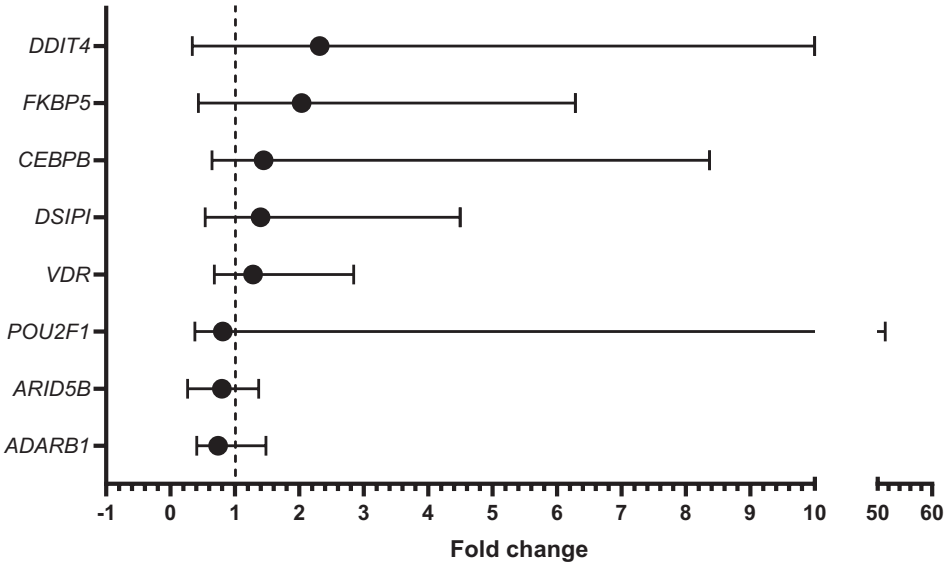

Figure 3. Gene expression fold change in normal cortisol relative to very low cortisol. The black circles mark the median fold change and the whiskers mark the range.

value of *POU2F1* has primarily been demonstrated in the setting of cancer, as measurement of POU2F1 protein levels is better than clinical cancer staging in predicting prognosis [34].

Finally, *ARID5B* has been associated with susceptibility to and treatment outcomes of acute lymphatic leukemia (ALL), and lower *ARID5B* expression at diagnosis has been linked to increased risk of ALL relapse [35]. To our knowledge, this is the first study to demonstrate the inhibitory effect of GCs on *ARID5B* expression.

One patient in step 1 was not cortisol deplete before HC infusion. We do not, however, doubt that the diagnosis of AAD was correct as all study patients were recruited from our national quality registry (the National Registry for Addison's disease [ROAS]) and were carefully characterized both clinically and biochemically. Furthermore, the patient had clearly elevated ACTH (198 pmol/L) before HC infusion that was successfully suppressed after HC infusion (15.1 pmol/L). We propose that this patient had residual GC production, which we recently demonstrated is present in one-third of all patients with AAD [36]. To ensure full privacy protection, all samples were anonymized before analysis, and we were unable to identify the study patient in order to verify residual GC production.

In step 2, the cross-over design allowed us to evaluate changes in gene expression in the same individuals at normal cortisol (the CSHI arm) compared to very low cortisol levels (the OHC arm). We are, however, aware that our results instead could represent changes in gene expression related to the 2 different treatment modalities, CSHI and OHC. This would, however, require that the half-life of gene expression (RNA) levels exceeded 15 hours, corresponding to the minimum length of medication fasting in the OHC arm. Although we do not know the exact half-life of all the included genes, the median half-life of gene expression in general is estimated to be 7.1 hours [37].

Finally, since this was an exploratory study, we acknowledge that interpretation of our data must be done with caution. In particular, without a control group of healthy individuals, we cannot know whether the observed differences in gene expression are changes toward a more normal physiological state or quite the reverse. In a future clinical study, we therefore advocate the need for a control group of healthy individuals to establish reference ranges for gene expression levels. Nevertheless, by presenting the data as-is, we provide a basis for future studies to explore the true potential and validity of gene expression as biomarkers for GC replacement in patients with AAD. Specifically, we suggest a repeated-measures design where gene expression levels are measured in the same individuals with AAD on repeated occasions following different GC administration types and doses, compared with healthy controls.

In conclusion, we nominate the gene expression of *DSIPI, DDIT4,* and *FKBP5* as candidate transcriptional biomarkers of GC replacement, followed by *CEBPB, VDR, POU2F1, ARID5B, ADARB1,* and *MMP9*.

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Data Availability: The datasets analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request. Supplementary results are available in the data repositories listed in References.

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