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Identification of glucocorticoid-related molecular signature by whole blood methylome analysis

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Abstract

Objective: Cushing's syndrome represents a state of excessive glucocorticoids related to glucocorticoid treatments or to endogenous hypercortisolism. Cushing's syndrome is associated with high morbidity, with significant inter-individual variability. Likewise, adrenal insufficiency is a life-threatening condition of cortisol deprivation. Currently, hormone assays contribute to identify Cushing's syndrome or adrenal insufficiency. However, no biomarker directly quantifies the biological glucocorticoid action. The aim of this study was to identify such markers.

Design: We evaluated whole blood DNA methylome in 94 samples obtained from patients with different glucocorticoid states (Cushing's syndrome, eucortisolism, adrenal insufficiency). We used an independent cohort of 91 samples for validation.

Methods: Leukocyte DNA was obtained from whole blood samples. Methylome was determined using the Illumina methylation chip array (~850000 CpG sites). Both unsupervised (Principal Component Analysis) and supervised (Limma) methods were used to explore methylome profiles. A Lasso-penalized regression was used to select optimal discriminating features.

Results: Whole blood methylation profile was able to discriminate samples by their glucocorticoid status: glucocorticoid excess was associated with DNA hypomethylation, recovering within months after Cushing's syndrome correction. In Cushing's syndrome, an enrichment in hypomethylated CpG sites was observed in the region of *FKBP5* gene locus. A methylation predictor of glucocorticoid excess was built on a training cohort and validated on two independent cohorts. Potential CpG sites associated with the risk for specific complications, such as glucocorticoid-related hypertension or osteoporosis, were identified, needing now to be confirmed on independent cohorts.

Conclusions: Whole blood DNA methylome is dynamically impacted by glucocorticoids. This biomarker could contribute to better assess glucocorticoid action beyond hormone assays.

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Introduction

Cushing's syndrome is a state of glucocorticoid excess related either to glucocorticoid treatment (exogenous Cushing's syndrome), or to excessive secretion of adrenocortical glucocorticoids. While effective in suppressing inflammatory states(1), the prolonged use of administered glucocorticoids is associated with potentially serious adverse effects, restricting their widespread and chronic usage(2). Clinical consequences of systemic glucocorticoid treatment are mirrored by states of endogenous cortisol excess. While overt manifestations of Cushing's syndrome are rare and most often related to pituitary adenomas(3), mild autonomous cortisol hypersecretion is more common and mostly caused by adrenal adenomas(4).

Cushing's syndrome is associated with high morbidity and mortality, and impaired quality of life(5) through numerous systemic manifestations, including diabetes mellitus, hypertension, osteoporosis, cutaneous bruising, muscular atrophy, neuropsychiatric disorders, and immune deficiency(6). Duration and level of glucocorticoid excess are undisputedly the main determinants of Cushing's syndrome severity. However, individual susceptibility highly affects the likelihood to develop each type of complication and modulates their severity(7).

On the other side of the clinical spectrum, adrenal insufficiency is a state of cortisol deprivation, inducing fatigue and acute decompensations of metabolism or electrolyte balance with potentially lethal outcome(8). Adrenal insufficiency can be caused by structural or functional damage of adrenal glands, pituitary or hypothalamus, with autoimmunity-, tumour- or treatment-related reasons as the most common causes.

Quantification of glucocorticoid action on peripheral tissues is difficult to assess by clinical means or on the basis of hormonal evaluations. Indeed, for exogenous glucocorticoid administration, pharmacokinetics may importantly influence the level of glucocorticoid excess, especially for low-dose systemic treatments, or in case of local administrations. For endogenous Cushing's syndrome, increased morbidity and mortality related to mild autonomous cortisol excess is well demonstrated on population level(9,10), but

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cannot be estimated properly on an individual level using classical hormone assays. For patients with adrenal insufficiency, titration of glucocorticoid supplementation relies mainly on clinical assessment. This shortcoming underlines the need for specific biomarkers quantifying glucocorticoid action, with potential impact on diagnosis, treatment decision and prediction of the individual risk for specific complications.

DNA methylation is a chemically stable yet dynamic biological hallmark, playing a key role in epigenetic regulation of gene expression in both health and disease(11). Several studies have suggested an association between hypothalamic-pituitary-adrenal axis dysregulation and specific blood DNA methylation profiles, particularly in post-traumatic stress disorders(12–16). In addition, the association between stress and DNA methylation has been explored for some targeted genes(17). Among them, methylation of *FKBP5* -encoding a co-chaperone of HSP90 protein involved in the regulation of glucocorticoid receptor activity(18)-, and *NR3C1* -encoding the glucocorticoid receptor-, are impacted by stress. Furthermore, a recent study showed a correlation between *FKBP5* expression and cortisol levels in patients with Cushing's syndrome(19).

The present study explores the impact of glucocorticoids on leukocytes methylation. Specifically, we analysed whole blood methylome in patients with endogenous Cushing's syndrome, eucortisolism or adrenal insufficiency, and we identified a methylome signature reflecting glucocorticoid excess.

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Materials and methods

Patients and samples

Ninety-four blood samples were collected from 47 patients with a confirmed diagnosis of endogenous Cushing's syndrome. Patients were followed in two expert centres, Cochin hospital (APHP, Paris, France) and LMU hospital (Ludwig-Maximilians-University, Munich, Germany). Diagnostic criteria of Cushing's syndrome included: increased 24h urine free cortisol, abnormal cortisol after 1 mg dexamethasone suppression and altered circadian cortisol rhythm, following consensus guidelines(20).

Blood samples were collected either before correction of Cushing's syndrome, or at least 3 months after (3 to 41 months; median: 12 months). At the time of blood sampling, patients were classified as overt Cushing's syndrome, mild Cushing's syndrome, eucortisolism or adrenal insufficiency, depending on clinical evaluation and hormone assays. Briefly, overt Cushing's syndrome patients presented clinical signs and increased 24h urine free cortisol (>240 nmol/24h), increased salivary midnight cortisol (>6 nmol/l) and insufficient cortisol suppression after 1mg dexamethasone (>50 nmol/l). Mild Cushing's syndrome patients lacked signs of clinically overt Cushing's syndrome, but had mild alterations of cortisol secretion, including either a slightly increased 24h urine free cortisol, or increased midnight salivary cortisol or insufficient cortisol suppression after 1mg dexamethasone. Adrenal insufficiency was based on low plasma morning cortisol (<160 nmol/l), and on insufficient response to corticotropin stimulation (<500 nmol/l). For two patients in eucortisolism, exact values were not available. Detailed hormone values for each sample are provided in Supplementary Table 1.

Signed informed consent for molecular analysis of blood samples and for access to clinical data was obtained from all patients, and the study was approved by a local Ethic Committee (for Cochin hospital: Comité de Protection de Personnes Ile de France 1, project 13495; for Munich: project 152-10).

Ninety-one additional samples were available and suitable for methylome analysis from patients enrolled in 5 specialized centers of the ENSAT-HT consortium (http://www.ensat-ht.eu). They included 26 patients

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with endogenous Cushing's syndrome(20) and 65 healthy volunteers (eucortisolism) (Supplementary Table 2). Signed informed consent was obtained from all patients, and the study was approved by the Ethic Committee of each participating center.

Whole-genome DNA methylation measurement

Leukocyte DNA was extracted from EDTA blood samples, using the DNA Isolation kit for Mammalian Blood (Roche, Basil, Switzerland). DNA quality was assessed on a Genomic DNA ScreenTape system (Agilent, Santa Clara, CA, US), and quantified using a Qubit 3.0 Fluorometer (Thermofisher, Waltham, MA, US). DNA was treated by bisulfite, then hybridized to the Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA, US; ~865,000 sites), starting from 500 ng of DNA. All experiments were performed following the manufacturer's instructions at the P3S Post-Genomic Platform of Sorbonne University (Paris, France).

Bioinformatics and statistics

All samples passed the quality controls provided by the Genome Studio software (v. 2011.1; Illumina). Data were exported as Intensity Data (IDAT) format, then processed using the minfi package (v. 1.32.0)(21) in R software environment (v. 3.6.3) (https://cran.r-project.org/).

Data were normalized using the stratified quantile normalization procedure implemented in the *preprocessQuantile* minfi function(22) and the methylation score for each CpG probe was extracted as a β-value. The ChAMP package (v. 2.16.1) was used to filter the probes(23). A total of 731,635 probes passed the following criteria: detection p-value<0.01, presence of the targeted CpG, absence of frequent SNPs in the probe, single hybridization hit, autosomal target.

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The significant components of variation in the dataset were assessed using the singular value decomposition method (SVD) for methylation data(24) and a detected batch effect (Slide) was corrected using the ComBat method(25), as implemented in the ChAMP package.

White blood cell count of subpopulations (neutrophils, lymphocytes B, lymphocytes T4, lymphocytes T8, lymphocytes NK, monocytes) were estimated by the reference-based RefbaseEWAS method(26) implemented in the ChAMP package. To confirm the reliability of the inferred white blood cell counts, we compared the estimated and available measured proportions of both neutrophils and lymphocytes, obtaining high correlation (Pearson's r=0.81 and r=0.87, respectively) (Supplementary Figure 1; Supplementary Table 3). Since neutrophils were the most represented cell type in all samples, and since the proportions of neutrophils and lymphocytes were negatively correlated (Pearson's r=-0.97), the estimated proportion of neutrophils was chosen as the unique proxy reflecting variations in white blood cell count.

M-values, used for statistical analyses, were calculated from β-values (log2 ratio of the intensities of methylated versus unmethylated probes) using the lumi package (v. 2.36.0)(27).

Global data structure was assessed on β-values by principal component analysis (PCA), using all CpG probes. Probe variability was calculated on M-values as standard deviation (SD) for each CpG site among samples. The most variable CpG probes (n=52,727 with SD>0.4) were selected for subsequent analyses.

Differentially methylated CpG sites were identified starting from the whole dataset using the Limma package (v. 3.40.6)(28), including the estimated neutrophils count as covariate, and considering a Benjamin-Hochberg adjusted p-value<0.05. Gene set enrichment analysis of genes associated with differentially methylated CpG sites was performed using the *gometh* method implemented in the *missmethyl* package (v. 1.18.0)(29), adjusting for the number of CpG sites associated to each gene (30). Differentially methylated regions were identified using the DMRcate package (v. 1.20.0)(31), comparing overt Cushing's syndrome versus eucortisolism or adrenal insufficiency, and using estimated neutrophil's

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count as covariate. Default parameters were applied to smooth the differential methylation signal and to define regions.

For predicting the glucocorticoid status from methylation, a training cohort of 60 samples was selected, randomly including 30 samples corresponding to overt Cushing's syndrome with no anti-cortisolic treatment at the time of sampling, and 30 samples corresponding to either eucortisolism or adrenal insufficiency after Cushing's syndrome treatment. Remaining samples (n=34) were used as a first validation cohort. CpG site selection was performed on the training cohort using a penalized Lasso regression on the most variable CpG probes (M-value SD>0.4), with a 10-fold cross-validation, using the glmnet package (v. 4.0-2)(32). The predictive model, including 29 discriminating CpG sites, was assessed on the validation cohort, graphically using a principal component analysis projection of samples based on the 29 CpGs methylation level, and statistically using an ordinal logistic regression model including a 29-CpGs predictor, calculated by adding the 29 CpGs M-values weighted by their Lasso coefficients. Similarly, the performance of the 29-CpGs methylation predictor was tested on the ENSAT-HT cohort, a second independent validation cohort.

In order to identify potential CpG sites specifically associated to glucocorticoid-related complications (hypertension, diabetes, osteoporosis), CpG site selection was performed on 47 Cushing's samples, starting from the most variable CpG probes (M-value SD>0.4), and using a penalized Lasso regression with a 10-fold cross-validation. A methylation predictor for each model was calculated as described for the 29-CpGs predictor.

Quantitative variable comparisons between groups were performed using two-tailed t-test or Wilcoxon's test, depending on variable distribution. Quantitative variable correlations were performed using Pearson's test. Multivariate analysis was performed using a logistic regression model including the 29-CpGs methylation predictor and the estimated proportion of neutrophils as covariates. All tests were computed in R software environment.

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Results

Cohort presentation

Ninety-four samples were collected from 47 patients with endogenous Cushing's syndrome (Table 1 and Supplementary Table 1). Median age was 46 years (range: 17 to 73), with a female predominance (1.8 to 1). Patients with endogenous Cushing's syndrome included Cushing's disease (n=39), benign adrenal Cushing's syndrome (n=7) and ectopic ACTH over-secretion (n=1). Cushing's syndrome-associated hypertension, diabetes, osteoporosis and catabolism – i.e., presence of either osteoporosis, osteopenia, muscle weakness, pigmented striae or other skin lesions- were present in 36 (77%), 16 (34%), 13 (28%) and 30 (64%) patients, respectively. Samples were collected at different time points during the course of the disease, thereby reflecting different states of glucocorticoid secretion: overt Cushing's syndrome (n=42), mild Cushing's syndrome (n=13), eucortisolism several months after Cushing's syndrome treatment (n=14), or adrenal insufficiency several months after Cushing's syndrome treatment (n=25). Samples were assigned either to training or to validation cohorts, as described in "Materials and methods" section.

An additional independent cohort of 91 samples, part of the European ENSAT-HT consortium, was collected, including 26 patients with endogenous Cushing's syndrome and 65 healthy volunteers (eucortisolism; Supplementary Table 2).

Glucocorticoid levels impact blood methylome

Whole genome blood DNA methylome was determined for the 94 samples, with 731,635 informative CpG sites in all samples. Unsupervised principal component analysis showed a discrimination of samples according to their glucocorticoid status, with a specific profile of overt Cushing's syndrome (Figure 1A). This discrimination was mainly related to the global methylation level. Indeed, overt Cushing's syndrome status was associated with overall decreased methylation among the most variable CpG sites (t-test p-

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value<0.05 for 52,727 CpG sites; Figure 1B). Another significant determinant was the white blood cell count variation (Supplementary Figure 2), related to the well-established effect of glucocorticoids on white blood cell composition, inducing granulocytosis and lymphopenia(33,34).

Exploration of glucocorticoid-related blood methylome profile

The specific effect of glucocorticoids on blood methylome was evaluated by comparing the methylation level in overt Cushing's syndrome samples (n=42) versus each of the other three groups individually – mild Cushing's syndrome (n=13), eucortisolism (n=14) and adrenal insufficiency (n=25) samples (Supplementary Table 4). The most significant difference was observed in the comparisons of overt Cushing's syndrome versus eucortisolism (n=1290 differentially methylated CpG sites) and overt Cushing's syndrome versus adrenal insufficiency (n=7120 differentially methylated CpG sites). Both comparisons showed a prevalence of hypomethylated CpG sites in overt Cushing's syndrome (80% and 73%, respectively). Hypomethylated CpG sites were observed both in "Open Sea" and "Island" regions, showing the independence of glucocorticoid-related hypomethylation from CpG enrichment in the genome (Figure 2A, Supplementary Figure 3A). Glucocorticoid-related hypomethylation was not related to any specific gene locus structure either (Figure 2B, Supplementary Figure 3B).

Gene set enrichment analysis of genes associated with the differentially methylated CpG sites in the two comparisons revealed an enrichment in immunity-related signalling pathways (Gene Ontology gene sets; FDR<0.05), particularly those relating to neutrophils degranulation (Supplementary Table 5 and 6).

Differentially methylated CpG sites were distributed all along the genome. One gene locus was strongly enriched in differential CpG sites, on chromosome 6, corresponding to the *FKBP5* gene locus (Figure 2C, Supplementary Figure 3C, Supplementary Table 4). Beyond the analysis of individual CpG sites, a specific analysis of differentially methylated regions identified 99 and 418 differentially methylated regions in overt Cushing's syndrome versus eucortisolism and versus adrenal insufficiency, respectively (Supplementary Tables 7 and 8). Again, one of the most significant differentially methylated regions

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associated with the *FKBP5* gene promoter (Stouffer's Z-score < 0.001) (Supplementary Table 7). This *FKBP5* promoter region included 5 CpG sites, whose methylation level properly discriminated overt Cushing's syndrome samples from all the others (t-test p-value<0.05; Figure 3A). This effect was tested on the ENSAT-HT cohort, confirming the lower methylation level of these *FKBP5*-associated CpG sites in Cushing's syndrome (Figure 3B). Since promoter methylation usually negatively correlates with gene expression(11), we measured *FKBP5* gene expression in a subset of 37 samples (14 overt Cushing's syndrome, 10 mild Cushing's syndrome, 7 eucortisolism, 6 adrenal insufficiency), for which whole blood RNA was available (Supplementary Methods). *FKBP5* expression was negatively correlated with the five *FKBP5* promoter-associated CpG sites (r = -0.55, p-value < 0.001), and positively with the 24h urine free cortisol (Pearson's r = 0.62, p-value < 0.001), demonstrating the potential interest of using *FKBP5* expression as a biomarker of glucocorticoid excess.

We next explored the kinetics of glucocorticoid-related methylome modification by comparing the methylation profile of four different samples available for one of the patients (patient P30), collected before, four days, seven months and thirty-five months after Cushing's syndrome correction. Hierarchical clustering of methylome profiles well discriminated the overt Cushing's syndrome sample. During the months following Cushing's syndrome correction, the three samples were properly ordered, showing a progressive overall re-increase of methylation (t-test p-value<0.05; Figure 4).

Predicting glucocorticoid status by blood DNA methylation

To select a limited set of CpG sites predicting the glucocorticoid status, we performed a Lasso-penalized linear regression on the training cohort, starting from the 52,727 most variable CpG sites. Twenty-nine CpG sites were selected (Supplementary Table 9), properly discriminating overt Cushing's syndrome in the training cohort, with confirmation in the validation cohort (Figure 5A). A 29-CpGs methylation predictor was generated by combining the M-values of the 29 selected CpG sites weighted by their Lasso

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coefficients. This predictor was significantly associated with the glucocorticoid status in the validation cohort (Odd's ratio: 1.58; 95% confidence interval: 1.25 to 2.08; p-value<0.001).

The 29-CpGs methylation predictor was then tested on the ENSAT-HT cohort, a second independent validation cohort (26 overt Cushing's syndrome and 65 eucortisolism samples). Samples were properly classified as Cushing's syndrome and eucortisolism respectively (Figure 5B), and the prediction value could be confirmed (Odd's ratio: 1.10; 95% confidence interval: 1.07 to 1.12; p-value<0.001), corresponding to an accuracy of 0.84.

One of the 29 selected CpG sites was located in the *FKBP5* gene locus. Among the CpG sites in this locus, the one with the highest correlation to the 29-CpGs methylation predictor (Pearson's r=-0.89) (Supplementary Table 10) properly discriminated on its own overt Cushing's syndrome from other samples (Supplementary Figure 4).

We finally tested to which extent the glucocorticoid effect on blood methylome was related to glucocorticoid-induced white blood cell count variations. In a multivariate model combining the 29-CpGs methylation predictor and the neutrophils proportion, the 29-CpGs methylation predictor remained significant (logistic regression p-value<0.001; Table 2).

Candidate CpG sites predicting Cushing's syndrome-related complications

In order to address whether blood DNA methylation is associated with specific glucocorticoid-related complications, we performed an exploratory Lasso regression analysis on Cushing's syndrome samples from the 47 patients. A combination of 4 CpG sites was able to discriminate Cushing's syndrome patients with and without hypertension. Similarly, 14 CpG sites discriminated patients with and without osteoporosis (Figure 6; Supplementary Tables 11 and 12). The combination of the methylation level of selected sites for hypertension and osteoporosis was not correlated with 24h urine free cortisol (Pearson's r=0.06 and r=-0.05 for hypertension and osteoporosis, respectively). No combination of CpG sites was able to discriminate Cushing's syndrome patients with and without diabetes.

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Discussion

In this study, we demonstrated that whole blood methylome quantified biological glucocorticoid action. This biomarker was able to discriminate glucocorticoid excess from eucortisolism and adrenal insufficiency, independently from hormone assays. This new insight may contribute to overcome common pitfalls in Cushing's syndrome diagnosis and management(35,36). In clinical practice, such a tool would be of limited benefit in case of overt Cushing's syndrome, when clinical signs and hormone assays straightforwardly establish the diagnosis. However, a non-hormonal biomarker, directly measuring glucocorticoid action, could particularly help in three conditions: (i) in patients with mild autonomous cortisol secretion, to decide between surveillance and surgical correction of glucocorticoid excess; (ii) in patients under local or low-dose systemic glucocorticoid treatments, to assess the global glucocorticoid level. Indeed, morning plasma cortisol is often low in these patients, and cannot properly assess the glucocorticoid level. This low glucocorticoid level may either reflect mild glucocorticoid excess with negative feedback on endogenous cortisol production, or adrenal insufficiency resulting from prolonged adrenal blockade(37); (iii) in patients with adrenal insufficiency, to determine the optimal glucocorticoid supplementation(38). At this stage, though, the performance of our biomarker in these intermediate conditions and in exogenous Cushing's syndrome remains to be established, as well as its clinical relevance.

Here, we have analysed the global methylation state of blood DNA. Blood DNA is easy to obtain and DNA methylation marks are robust and convenient to investigate. In addition, DNA methylation is highly variable, enabling its use as a suitable biomarker.

Our analysis revealed a global DNA hypomethylation signature associated with endogenous Cushing's syndrome, demonstrating the direct impact of glucocorticoids on DNA methylation. DNA hypomethylation was already observed in an experimental model of mice treated with exogenous glucocorticoids(16). We could delineate a methylation gradient reflecting the relative degree of glucocorticoid excess, ranging from overt Cushing's syndrome, to mild Cushing's

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syndrome/eucortisolism, and adrenal insufficiency. Of note, this signature derives from whole blood, an admixture of various cell types with potentially cell-dependent methylation patterns. Indeed, glucocorticoids have a direct effect on white blood cell count, inducing neutrophils increase and lymphocytes decrease(33,34). However, we could precisely infer white blood cell count from methylome profiles for each patient, and demonstrate that methylome prediction of glucocorticoid status remained significant after adjustment on white blood cell composition, and therefore that methylome profiles variations do not only reflect blood composition variations. In addition, the methylome signature provided here in terms of differentially methylated CpG sites and regions, is adjusted for white blood cell composition, thus focusing on differences not related to white blood cell composition. This suggests a global impact of glucocorticoids on methylation of some DNA regions, irrespective of their tissue of origin.

After correction of glucocorticoid excess, long-term consequences have been reported(39–42). An easily measurable biomarker reflecting the dynamic of biological changes in time, such as blood DNA methylation, could help monitoring patients during follow-up. We observed that blood DNA hypomethylation progressively recovers in the years following remission. Similarly, a subtle DNA hypomethylation was observed by *Glad et al.* several years after Cushing's syndrome correction(43). The authors could correlate some blood methylation levels at specific genomic regions with long-term Cushing-associated neuropsychological sequels. Whether blood DNA methylation can properly help to monitor recovery after Cushing's syndrome correction remains to be specifically explored.

In case of eucortisolism and mild Cushing's syndrome, our methylation predictor showed intermediate classification, between overt Cushing's syndrome and adrenal insufficiency, sometimes discrepant from clinical appraisal. In such conditions, it was not possible to assess whether the methylation predictor was more accurate than clinical evaluation. In such cases, the most relevant judgmental criteria would be the correlation with long-term complications. Therefore, we explored the association between methylation and some well-appreciated glucocorticoid-related complications. Some selected CpG sites were identified as

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associated with hypertension and osteoporosis. New cohorts would be necessary to validate this association. In addition, whether these markers are specific to glucocorticoid excess remains to be established in prospective trials. Indeed, the inclusion of patients with no glucocorticoid excess would serve as negative controls, necessary for discarding markers of hypertension, diabetes or osteoporosis not related to glucocorticoid excess.

Whole genome methylome profiling is not easily achievable in clinical routine, thus representing a limitation in using this new marker. A technology transfer to targeted methylation assays would be required, such as pyrosequencing, methylation specific-MLPA, or methylation specific-high resolution melting analysis(44). An alternative would be the identification of surrogate DNA regions recapitulating this global information. One region could be represented by the *FKBP5* gene locus, whose methylation and expression have been demonstrated to be modulated by glucocorticoids in different tissues(45–50), with hypomethylation of the promoter region associated with increased gene expression. In this study, we also identified the *FKBP5* promoter region methylation as strongly associated with glucocorticoid excess, and negatively correlated with *FKBP5* gene expression. Particularly, one single CpG site from the *FKBP5* gene locus could discriminate overt Cushing's syndrome samples.

Sample size is another limitation of this study, with 94 samples from 47 patients. However, samplings were performed at different times of the disease, corresponding to different glucocorticoid statuses. Additional samples from the ENSAT-HT cohort allowed to further validate the performance of our marker. Further extending the cohort would help to confirm this finding, helping to better characterize the association with specific complications of glucocorticoid excess.

In conclusion, glucocorticoids induce a dynamic whole blood DNA methylome signature. This signature could be used as a biomarker for assessing glucocorticoid action independently from hormone assays.

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Declaration of interest, Funding and Acknowledgements

Declaration of interest

The authors declare no conflict of interest.

Guillaume Assié is on the editorial board of EJE. Guillaume Assié was not involved in the review or editorial process for this paper, on which he/she is listed as an author.

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Author contributions

R.A., G.A.: conceptualization; L.B., L.B., A.R., F.B.S., A.B., L.A., C.S., F.C., G.P.R., T.A.W., C.K.L., F.B., M.R., J.B., G.A.: clinical data and sample collection; M.C.Z., M.R., F.B., J.B., G.A.: project administration and ethical aspects management; R.A., C.G., K.P.: samples handling and genomic data generation; R.A., A.J., A.S., T.L., M.N., S.A., G.A.: bioinformatics and statistical analyses; R.A., G.A.: original draft preparation. All authors: manuscript review and editing.

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Data access

Methylome data generated in this study have been deposited in the ArrayExpress database at EMBL-EBI (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10092).

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Figure legends

Figure 1. Glucocorticoid levels impact on whole blood DNA methylation. A) Samples projection based on the two principle components (PC1, PC2) of unsupervised PCA performed on the whole dataset (n=731,635 CpG sites, n=94 samples). B) Representation of global methylation (median M-value) relative to the most variable CpG sites (n=52,727 with a M-value standard deviation>0.4) in the four groups. *p-value<0.05, **p-value<0.001, ***p-value<10^{-10}.

Figure 2. Distribution of differentially methylated CpG sites (overt Cushing's syndrome vs. eucortisolism: n=1290). A) Distribution relative to genome CpG enrichment. B) Distribution relative to gene locus structure. C) Genomic distribution. Highlighted in black, the CpG sites located in the *FKBP5* gene locus on chromosome 6.

Figure 3. Methylation levels of the *FKBP5* promoter region in Cushing's syndrome samples.

A) Boxplot representation of the mean methylation (M-value) of the 5 CpG sites included in the differentially methylated region associated to the *FKBP5* gene promoter, in the principal cohort. B) Boxplot representation of the methylation level of the same 5 CpG sites in the ENSAT-HT cohort. **p-value<0.001, ***p-value<10^{-5}.

Figure 4. Kinetics of methylome modifications after normalization of glucocorticoid excess. Unsupervised clustering of four samples from patient P30, collected before and at three different time points after Cushing's syndrome correction. The mean methylation (M-value) of the 7426 CpG sites differentially methylated in overt Cushing's syndrome is provided below. ***p-value<10^{-15}.

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Figure 5. Discrimination of samples based on the 29-CpGs methylation predictor. A) Samples projection based on the two principle components (PC1, PC2) of unsupervised PCA performed using the 29-CpG sites selected by Lasso regression on the training cohort. In faint circles are presented the samples from the training cohort, on which the optimization of CpG selection was operated. In bright squares are presented the samples from the validation cohort. B) Similar projection using a second independent validation cohort, with samples from the ENSAT-HT cohort presented in bright triangles.

Figure 6. Discrimination of glucocorticoid-related complications. Projection of the 47 Cushing's syndrome samples based on the two principle components (PC1, PC2) of unsupervised PCA performed using the CpG sites selected by Lasso regression discriminating hypertension (4 CpGs, panel A) and osteoporosis (14 CpGs, panel B).

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Table 1 Characteristics of the samples tested. Cortisol values are provided as median values with ranges.

Glucocorticoid Status	Normal range	Global cohort	Training cohort	Validation cohort	*P*-value*
Total number of samples		94	60	34	
Overt Cushing's syndrome					
n		42	30	12	
Urinary free cortisol, nmol/24h	<240	1163 [306-44375]	1907 [306-44375]	896 [329-3496]	0.021
Midnight salivary cortisol, nmol/l	<6	20 [6-194]	22 [6-194]	13 [6-97]	0.046
Plasma cortisol after 1mg DST, nmol/l	<50	377 [74-1883]	400 [74-1883]	293 [110-822]	0.155
Mild Cushing's syndrome					
n		13		13	
Urinary free cortisol, nmol/24h	<240	213 [68-360]		213 [68-360]	
Midnight salivary cortisol, nmol/l	<6	10 [3-17]		10 [3-17]	
Plasma cortisol after 1mg DST, nmol/l	<50	64 [32-215]		64 [32-215]	
Eucortisolism					
n		14	8	6	
Urinary free cortisol, nmol/24h	<240	188 [71-304]	125 [97-276]	207 [71-304]	0.690
Midnight salivary cortisol, nmol/l	<6	4 [1-11]	3 [1-5]	5 [2-11]	0.167
Plasma cortisol after 1mg DST, nmol/l	<50	37 [25-48]	37 [30-48]	35 [25-44]	0.8
Adrenal insufficiency					
n		25	22	3	
Early morning plasma cortisol, nmol/l	160-500	83 [6-287]	82 [6-287]	97 [17-218]	0.645
Cortisol after ACTH stimulation, nmol/l	>500	276 [19-1322]	331 [19-1322]	91 [41-1092]	0.616

*Wilcoxon's test comparing training and validation cohorts.

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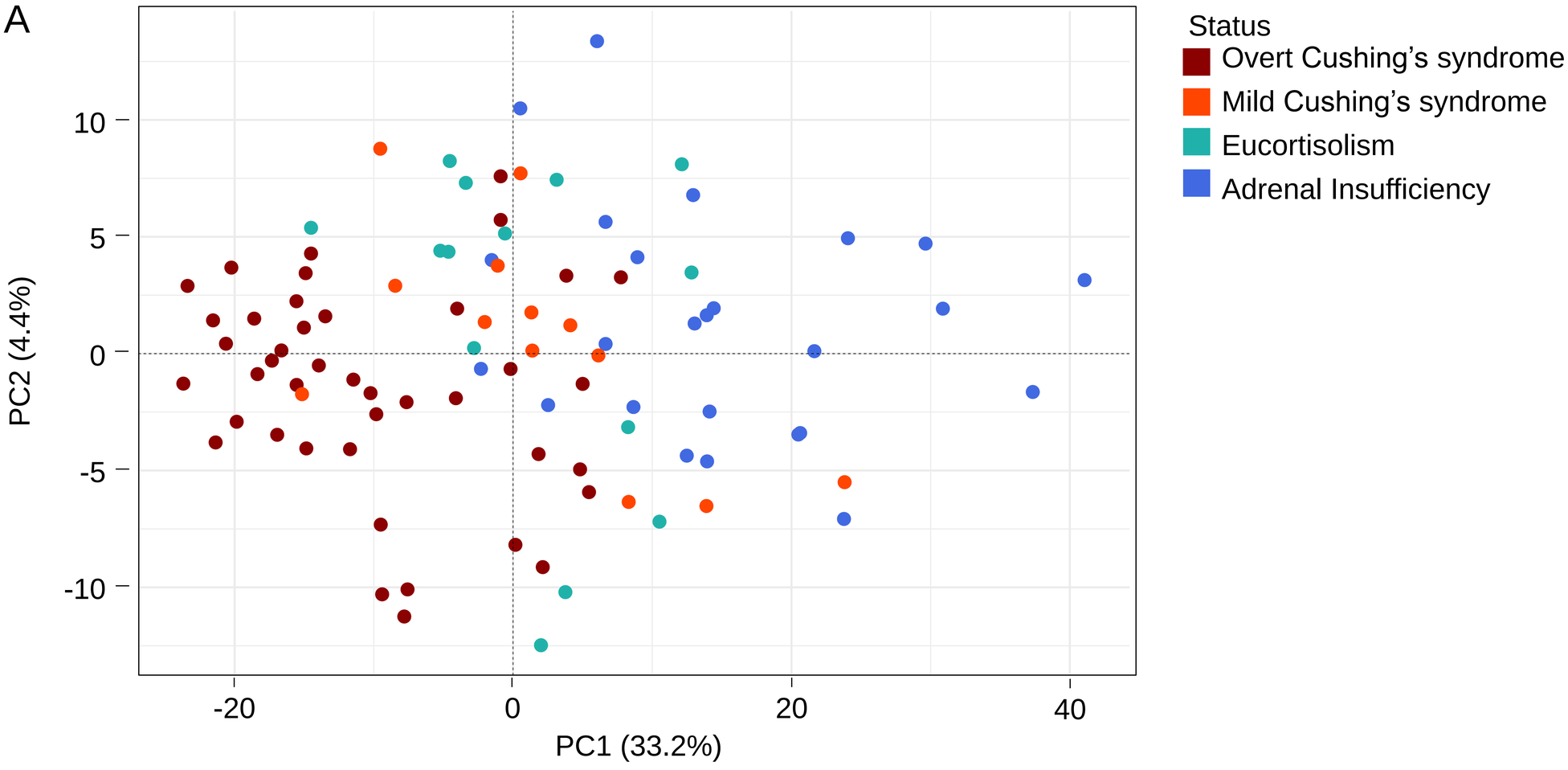
Table 2

Variables	OR	95% CI	p-value
29-CpGs methylation predictor	2.02	1.51 – 3.0	<0.001
Proportion of neutrophils (%)	0.97	0.87 – 1.08	0.6

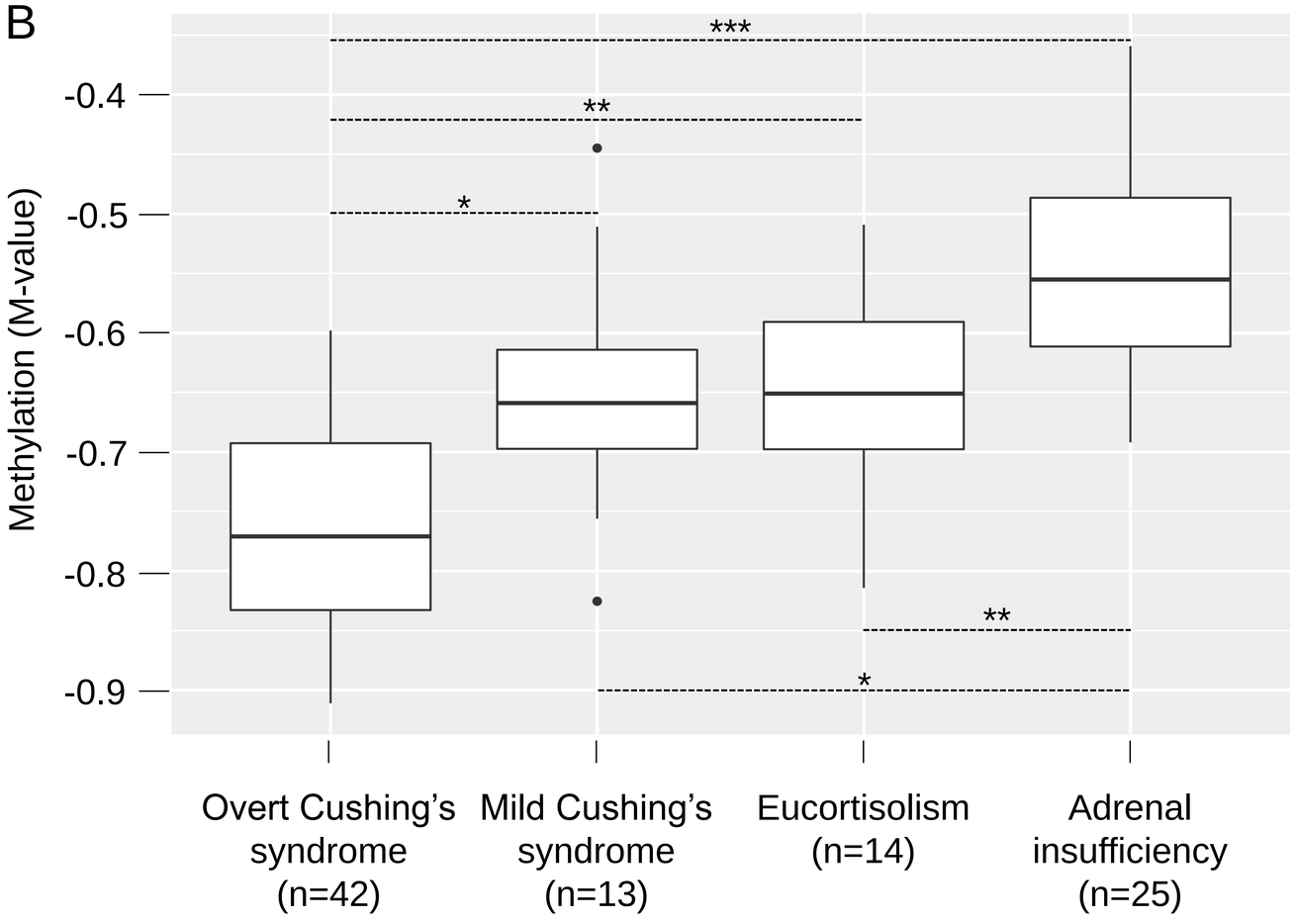
Table 2. Multivariate model combining methylome and neutrophils predictors on glucocorticoid status. Two statuses were considered: Cushing's syndrome (overt or mild) and not Cushing's syndrome (eucortisolism or adrenal insufficiency). OR = Odds ratio, CI = Confidential Interval.

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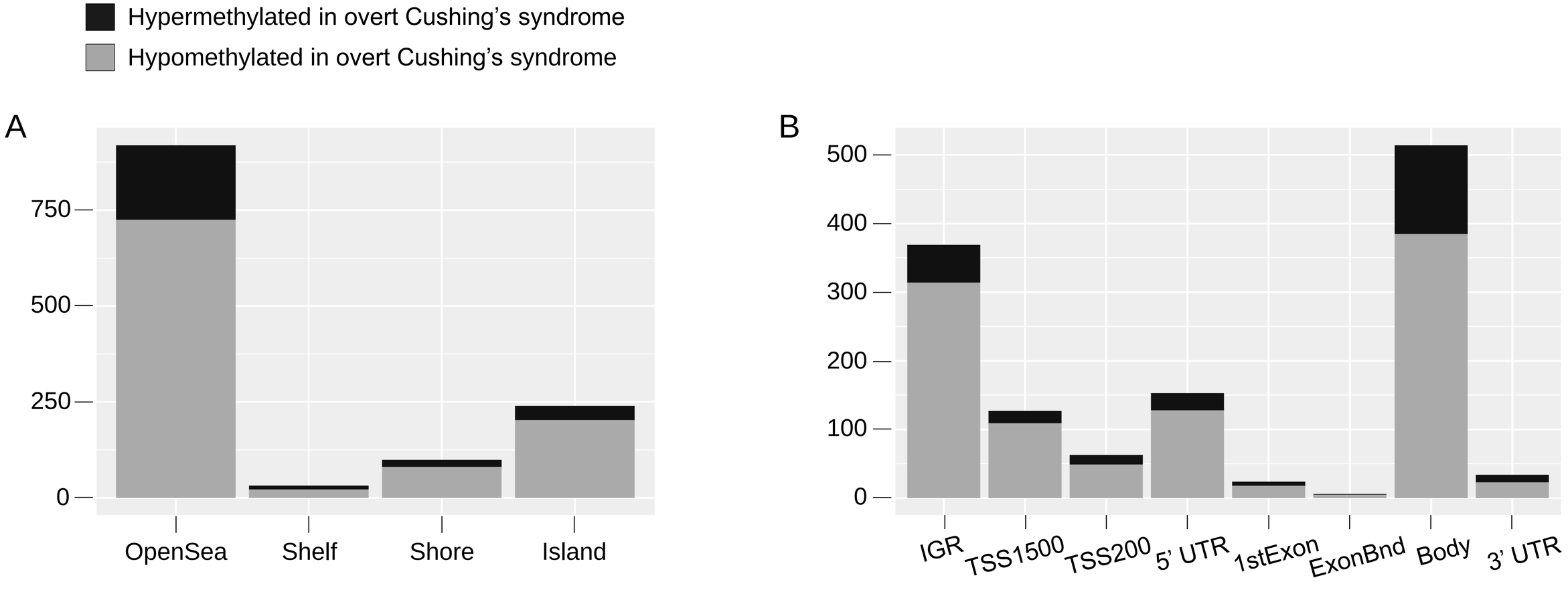
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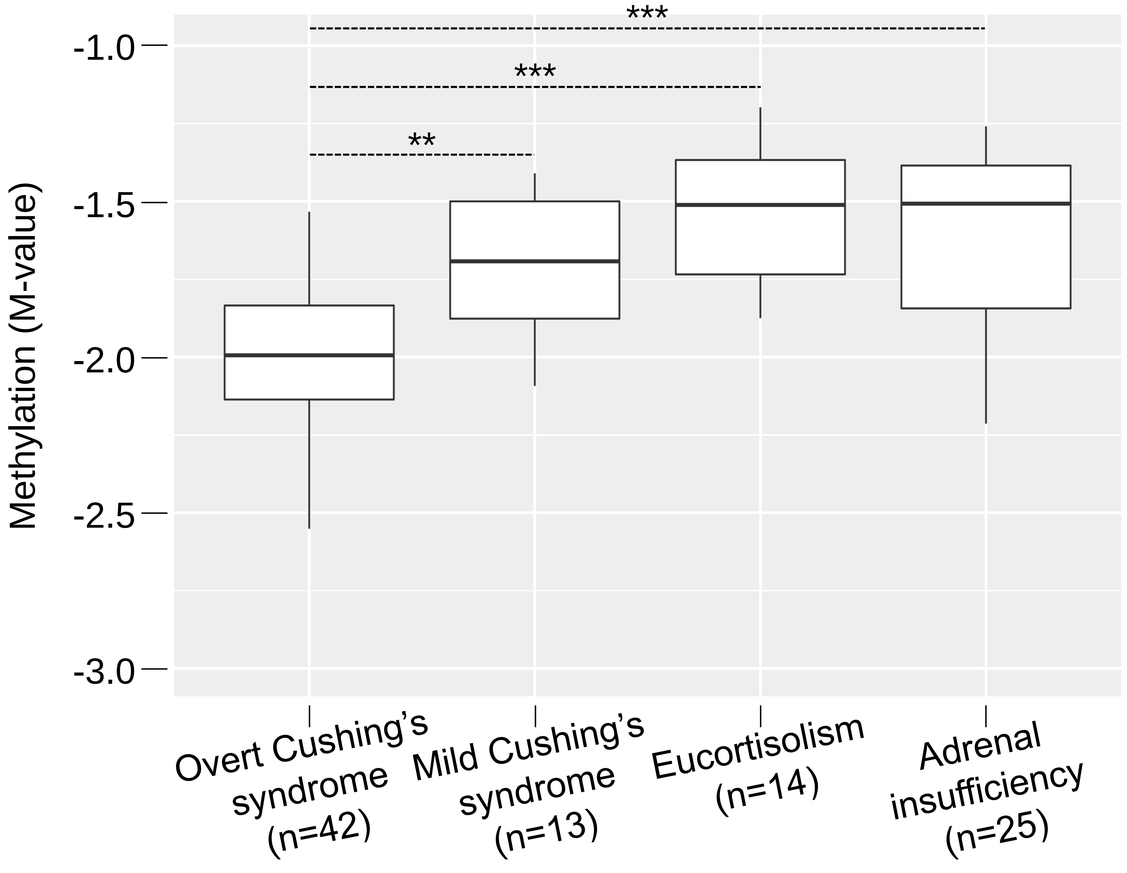
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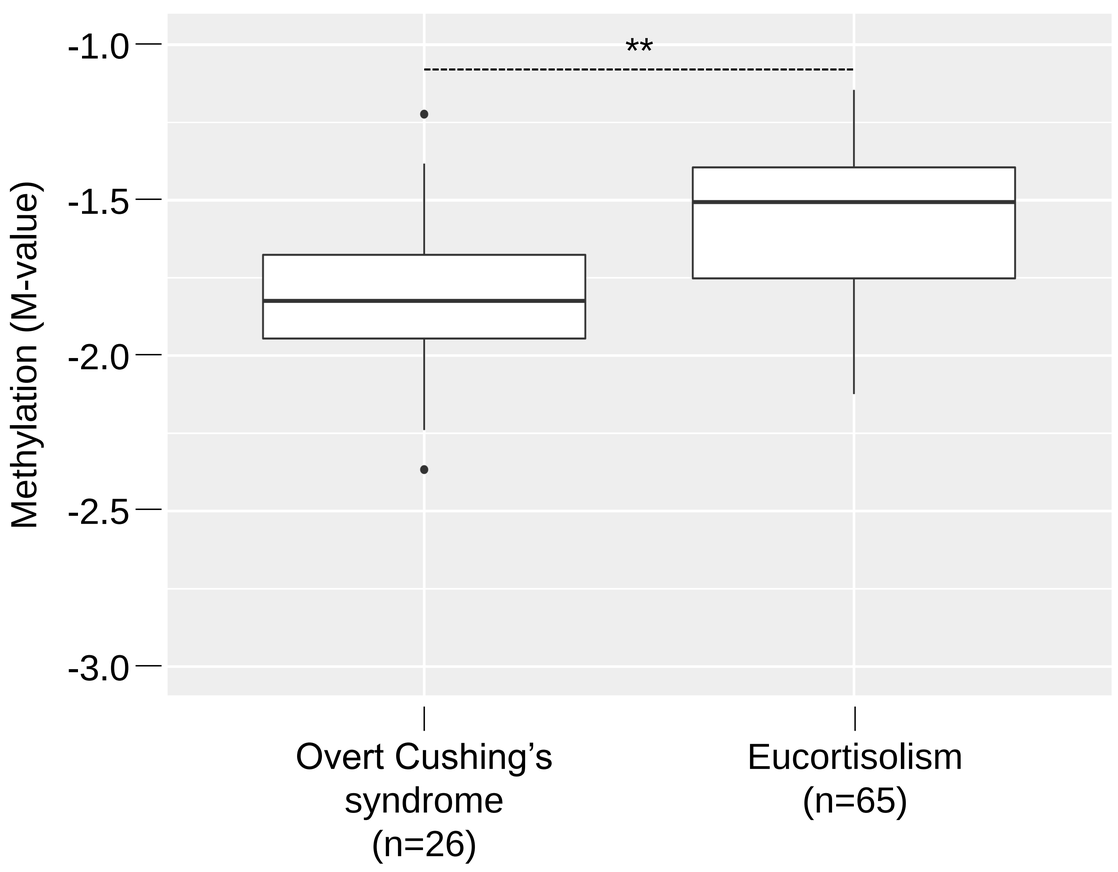
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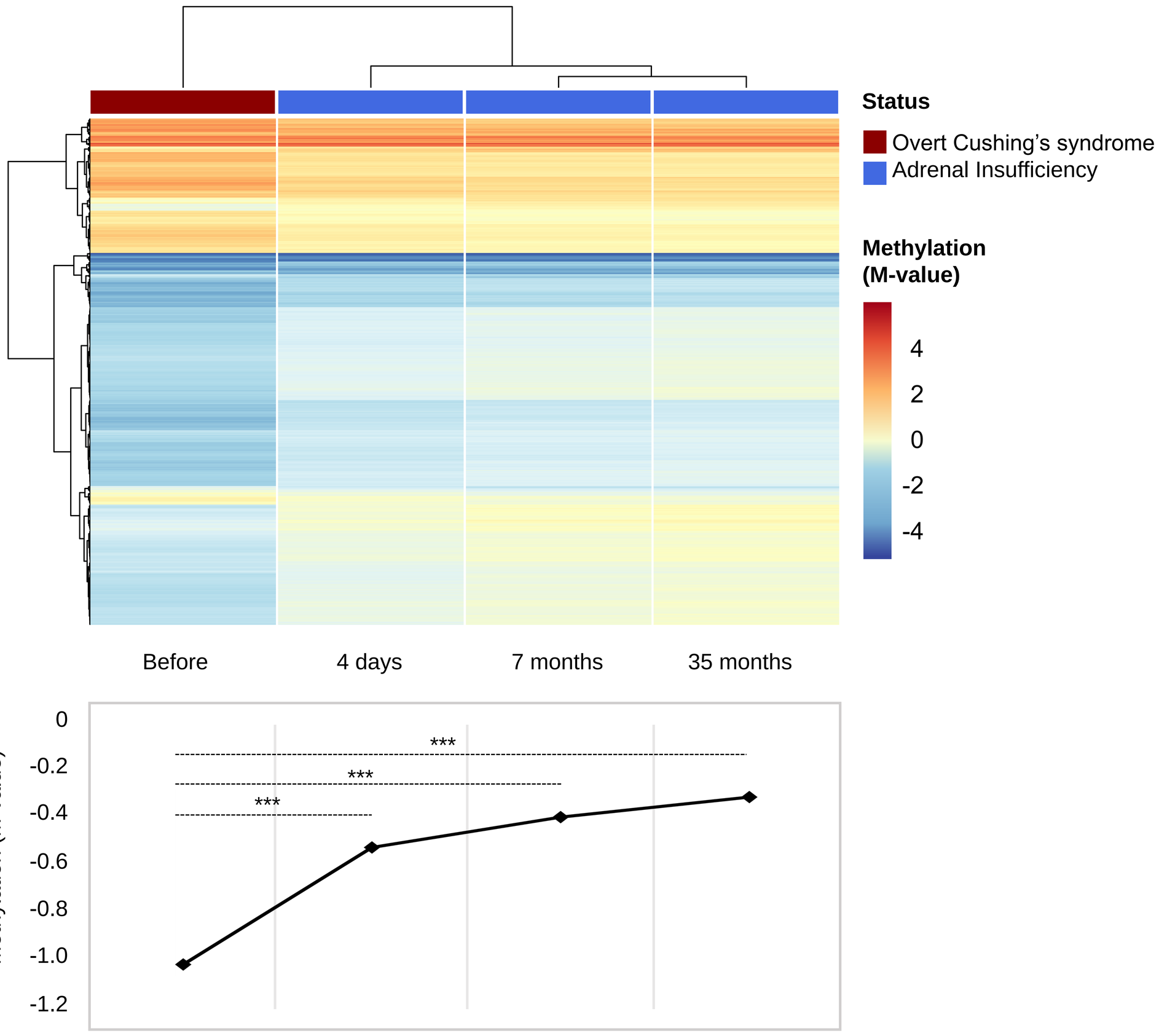
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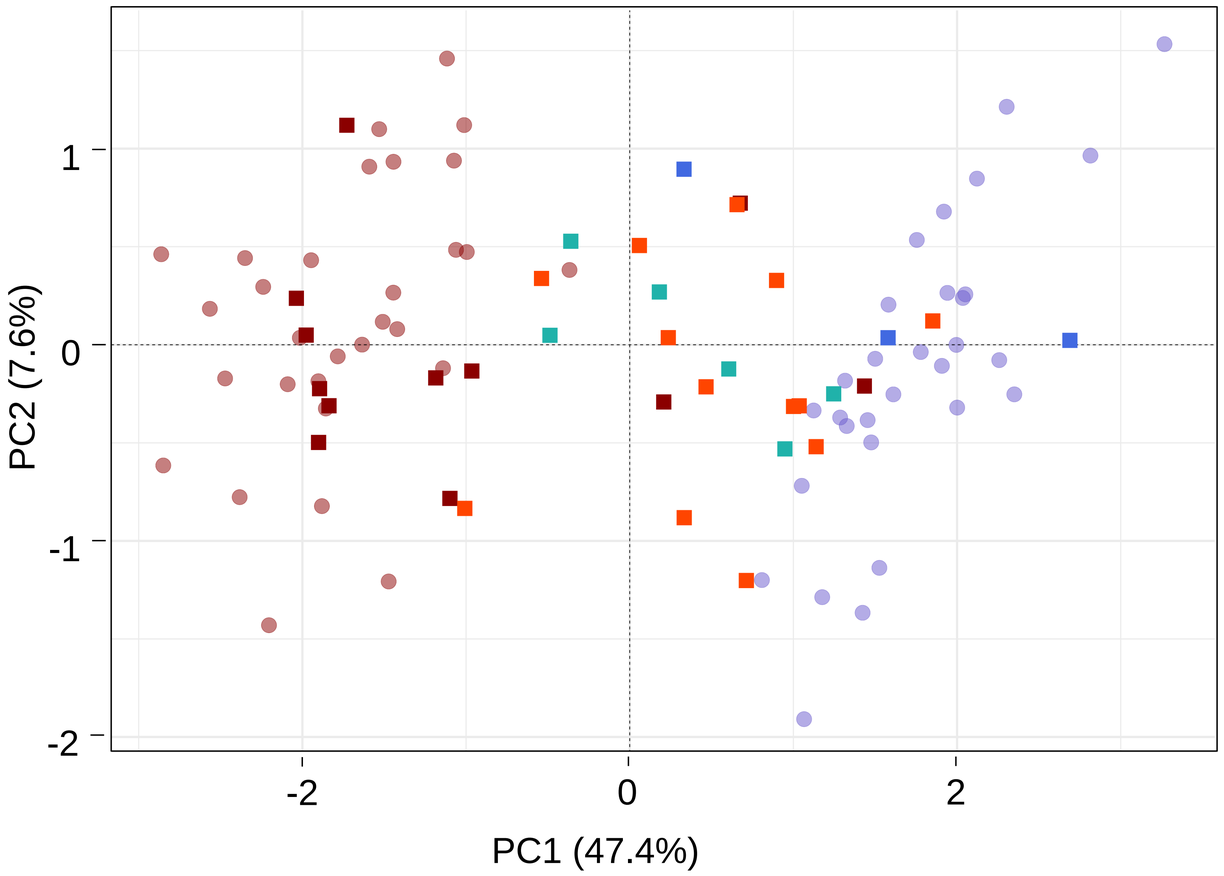
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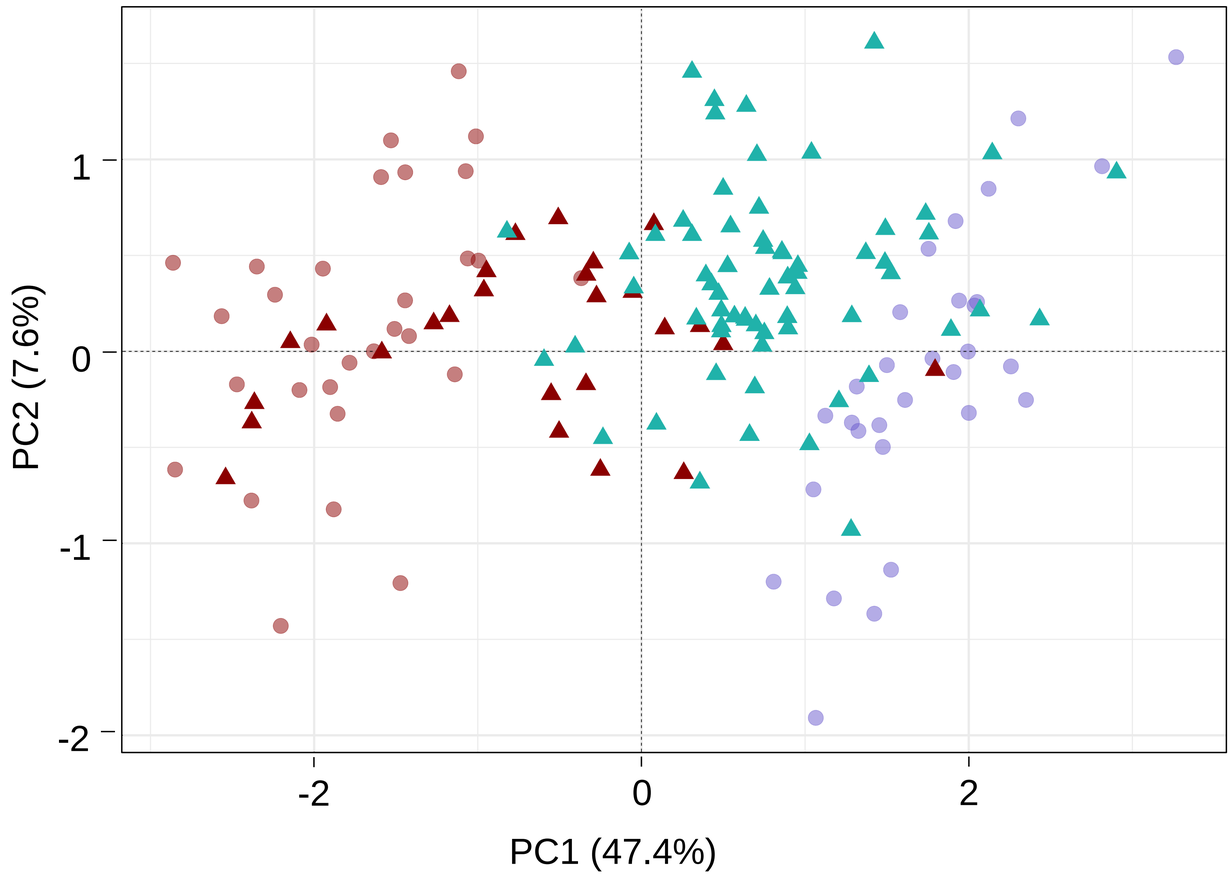
Status – Training cohort

- Overt Cushing's syndrome
- Eucortisolism/Adrenal Insufficiency

Status – Validation cohort

- Overt Cushing's syndrome
- Mild Cushing's syndrome
- Eucortisolism
- Adrenal Insufficiency

B



Status – Training cohort

- Overt Cushing's syndrome
- Eucortisolism/Adrenal Insufficiency

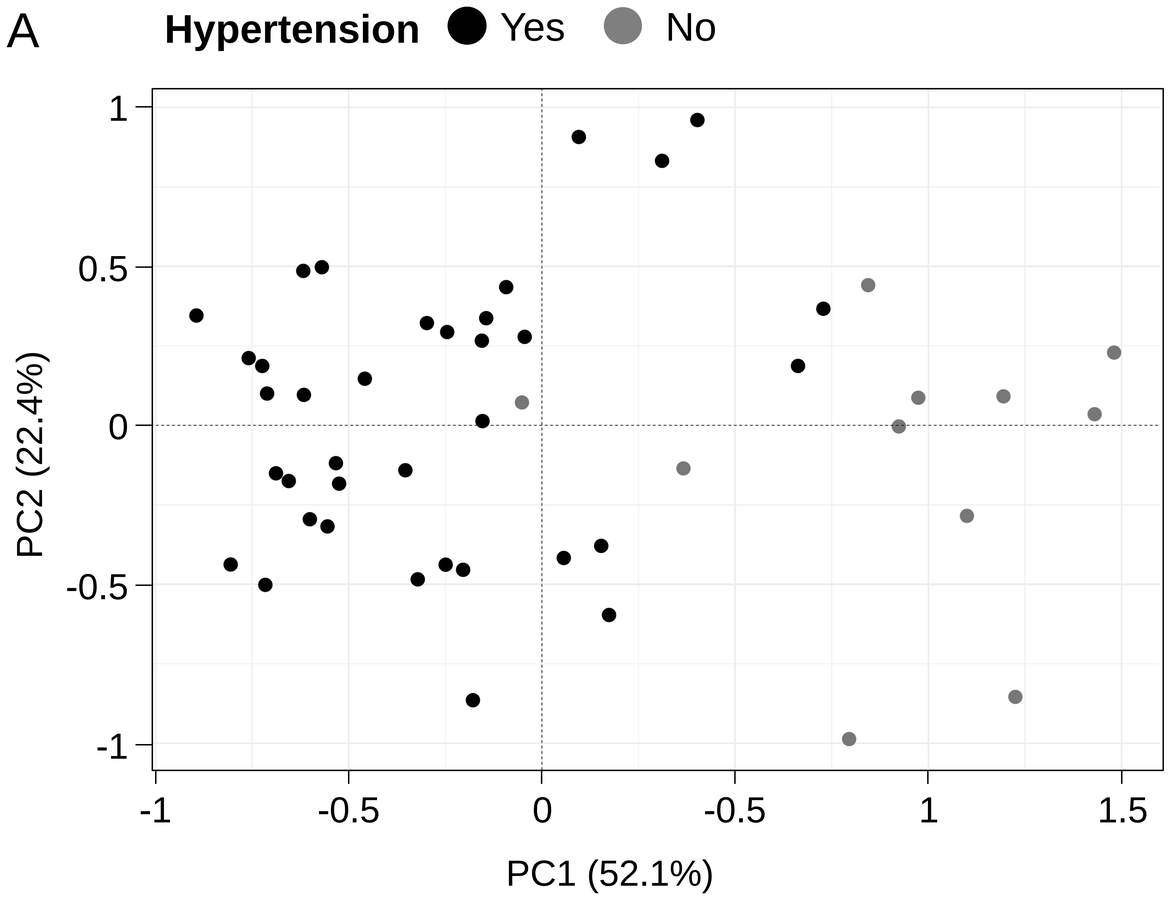
Status – ENSAT-HT cohort

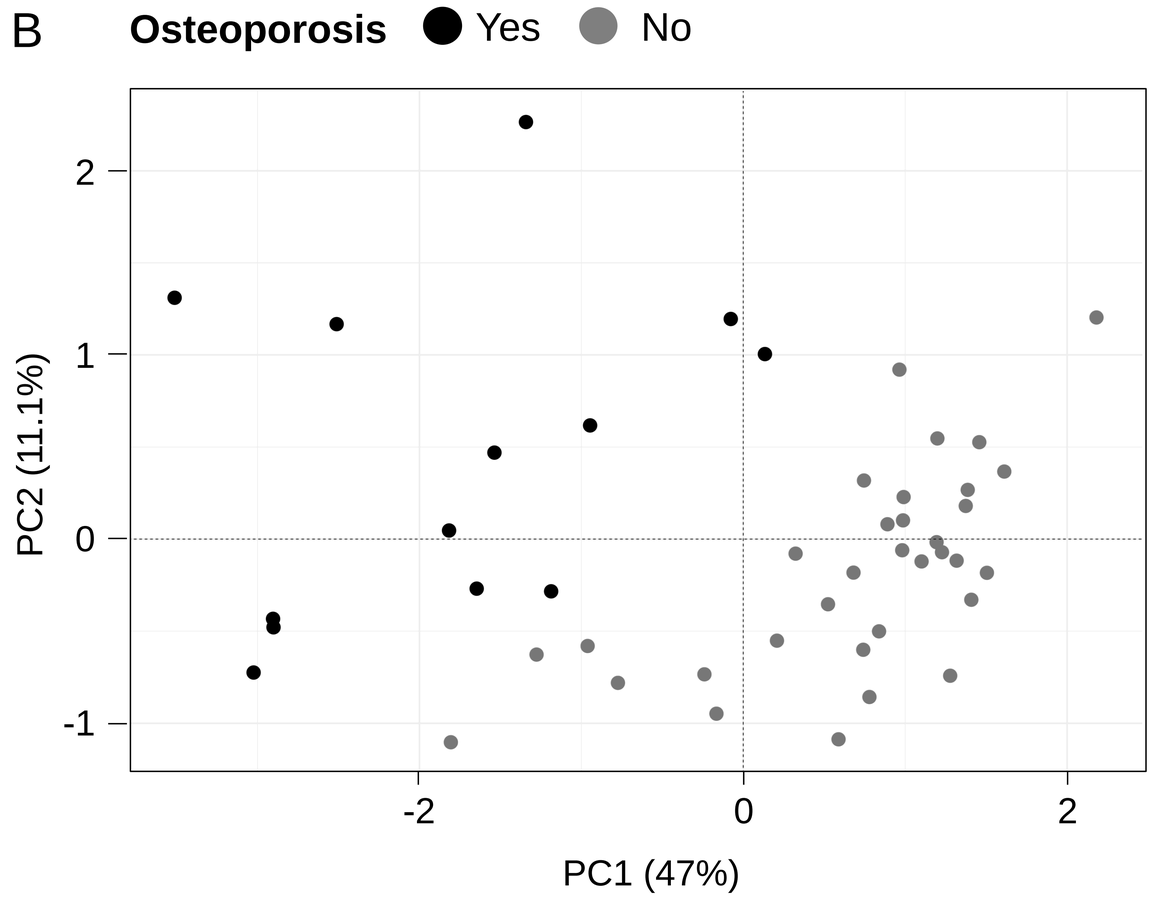
- Overt Cushing's syndrome
- Eucorticolism

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Supplementary Methods

RNA extraction and RT-qPCR

Total RNA was extracted from whole blood samples, collected into PAXgene tubes (PreAnalytiX, Hombrechtikon, Switzerland), by using a specific extraction kit (Qiagen, Hilden, Germany). The expression levels of target genes were determined by means of real-time PCR using a LightCycler Fast Start SYBR Green kit (Roche, Basil, Switzerland) according to the manufacturer's instructions. Relative quantification of target cDNA was determined by calculating the difference in cross-threshold (CT) values after normalization to *PPIA* (Cyclophilin A) signals (ΔΔCT method). Primer sequences for target genes were the following: for *FKBP5*, 5'AAGAGTGGGGAATGGTGAGG (Forward primer) and 5'ATGGTAGCCACCCCAATGTC (Reverse primer); for *PPIA*, 5'ATGGCACTGGTGGCAAGTCC (Forward primer) and 5'TTGCCATTCCTGGACCCAAA (Reverse primer). Amplification was performed at 60°C.

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Supplementary Tables

Supplementary Table 1 – Samples characteristics

"Supplementary_Table1.xlsx" file

Supplementary Table 2 – ENSAT-HT independent cohort samples

"Supplementary_Table2.xlsx" file

Supplementary Table 3 – Proportion of measured and estimated neutrophils and leukocytes

"Supplementary_Table3.xlsx" file

Supplementary Table 4 – Significant differentially methylated CpG sites in overt Cushing's syndrome

"Supplementary_Table4.xlsx" file

Supplementary Table 5 – Gene set enrichment analysis: overt Cushing's syndrome versus eucortisolism

"Supplementary_Table5.docx" file

Supplementary Table 6 – Gene set enrichment analysis: overt Cushing's syndrome versus adrenal insufficiency

"Supplementary_Table6.docx" file

Supplementary Table 7 – Differentially methylated regions: overt Cushing's syndrome versus eucortisolism

"Supplementary_Table7.xlsx" file

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Supplementary Table 8 – Differentially methylated regions: overt Cushing's syndrome versus adrenal insufficiency

"Supplementary_Table8.xlsx" file

Supplementary Table 9 - 29-Lasso selected CpG sites

"Supplementary_Table9.docx" file

Supplementary Table 10 – *FKBP5* gene locus-associated CpG sites

"Supplementary_Table10.xlsx" file

Supplementary Table 11 – Hypertension-associated CpG sites

"Supplementary_Table11.docx" file

Supplementary Table 12 – Osteoporosis-associated CpG sites

"Supplementary_Table12.docx" file

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Supplementary Figures

Supplementary Figure 1. Correlation between measured and estimated neutrophils (A) and lymphocytes (B).

Supplementary Figure 2. Components of variation in the whole methylome dataset. A) Scree plot representing the percentage of explained variability by the first five principal components of PCA performed on the whole dataset (n=731,635 CpG sites, n=94 samples). PC-1 accounts for most of the variability. B) Singular value decomposition (SVD) plot assessing the correlation between the first five significant components of variation in the dataset and biological factors of interest (Status –overt Cushing's syndrome, mild Cushing's syndrome, eucortisolism, adrenal insufficiency-, age, sex, proportion of neutrophils).

Supplementary Figure 3. Distribution of differentially methylated CpG sites (overt Cushing's syndrome vs. adrenal insufficiency: n=7120). A) Distribution relative to genome CpG enrichment. B) Distribution relative to gene locus structure. C) Genomic distribution. Highlighted in black, the CpG sites located in the *FKBP5* gene locus on chromosome 6.

Supplementary Figure 4. Methylation level of one single CpG site from the *FKBP5* gene locus in Cushing's syndrome samples. A) Boxplot representation of cg19226017 methylation level, a CpG site belonging to the *FKBP5* promoter region and showing the highest correlation with the 29-CpG methylation predictor. B) Boxplot representation of the methylation level of the same *FKBP5*-associated CpG site -cg19226017- in the ENSAT-HT cohort. ***p-value<10^{-5}.

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1 **Supplementary Table 11 – Hypertension-associated CpG sites**

CpG name	Chromosome	Genome position (GRCh37)	Islands Name	Relation to Island	Gene Name	Gene Locus	Lasso coefficient
cg01967073	chr7	48147130		OpenSea	UPP1	Body	-0.12
cg24649335	chr3	14845410		OpenSea			0.07
cg01884612	chr22	31031439	chr22:31031358-31032059	Island	SLC35E4	TSS1500	-0.01
cg03380349	chr16	47492751	chr16:47494697-47495207	N_Shore	ITFG1	5'UTR	-1.14

2

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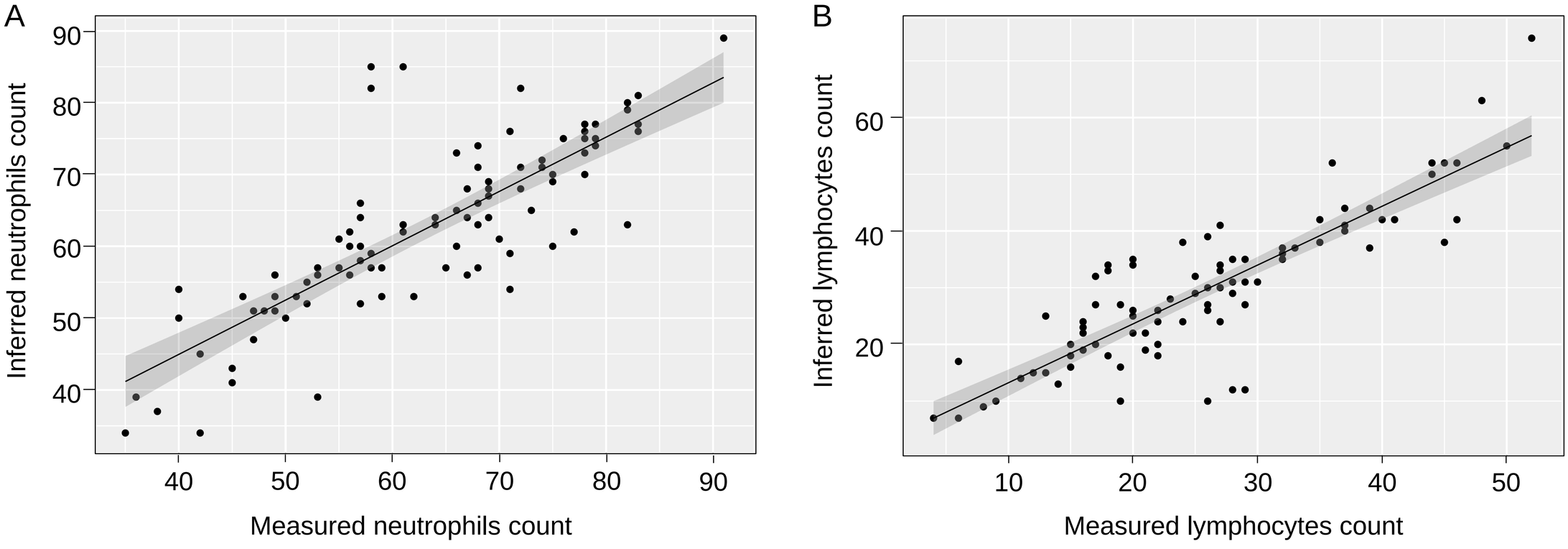
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1 **Supplementary Table 12 – Osteoporosis-associated CpG sites**

CpG name	Chromosome	Genome position (GRCh37)	Islands Name	Relation to Island	Gene Name	Gene Locus	Lasso coefficient
cg16611967	chr16	90144006	chr16:90143683-90144081	Island			0.22
cg00524694	chr5	177387682	chr5:177388753-177389250	N_Shore			-0.05
cg01629329	chr20	2633258	chr20:2632678-2633690	Island	NOP56	1stExon	0.50
cg10563109	chr6	5087749	chr6:5084516-5087032	S_Shore			-0.09
cg04160749	chr8	58172571	chr8:58172076-58173863	Island			0.13
cg12912919	chr7	92079392	chr7:92076699-92077395	S_Shore	GATAD1	Body	0.51
cg09641660	chr1	28198869	chr1:28199031-28199257	N_Shore	THEMIS2	TSS200	0.51
cg19137662	chr20	2633362	chr20:2632678-2633690	Island	NOP56	Body	0.04
cg07789658	chr8	23386185	chr8:23386220-23387386	N_Shore	SLC25A37	TSS200	0.49
cg19174044	chr20	18446362	chr20:18447429-18448341	N_Shore	DZANK1	5'UTR	0.14
cg05880330	chr3	137484517	chr3:137482964-137484454	S_Shore			0.16
cg13557804	chr6	33117976		OpenSea			0.04
cg23603273	chr10	64679766		OpenSea			-0.05
cg19623588	chr2	61149856		OpenSea	REL	3'UTR	-0.24

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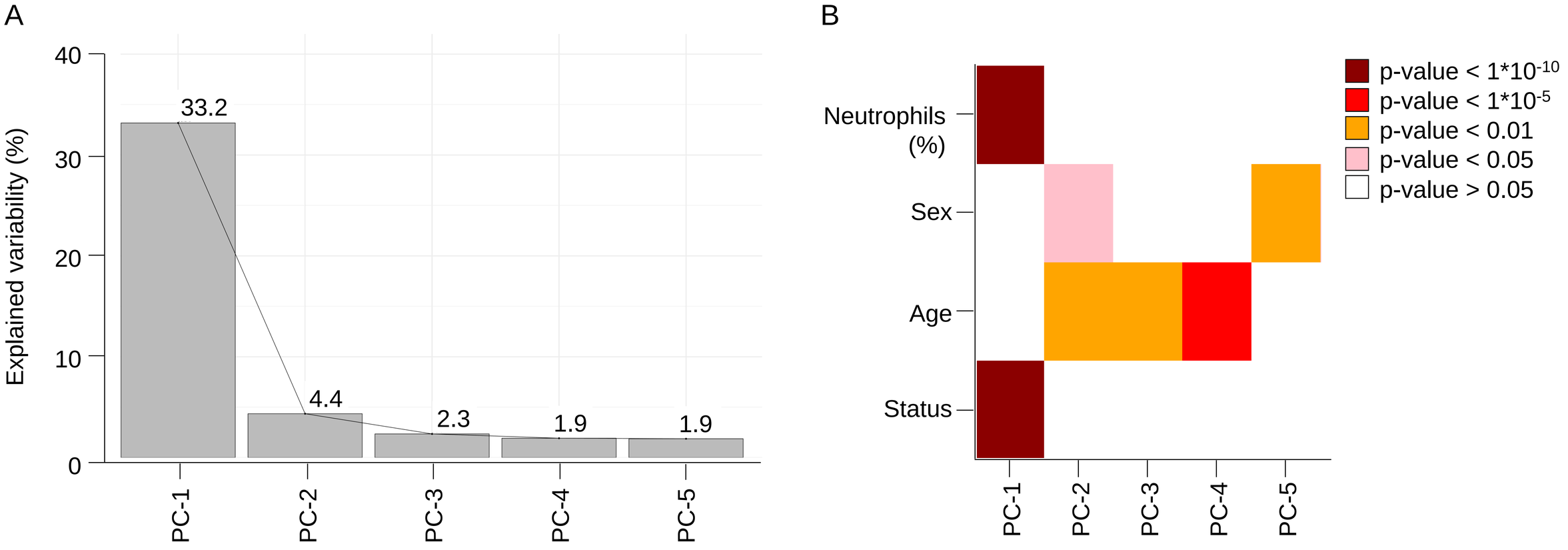
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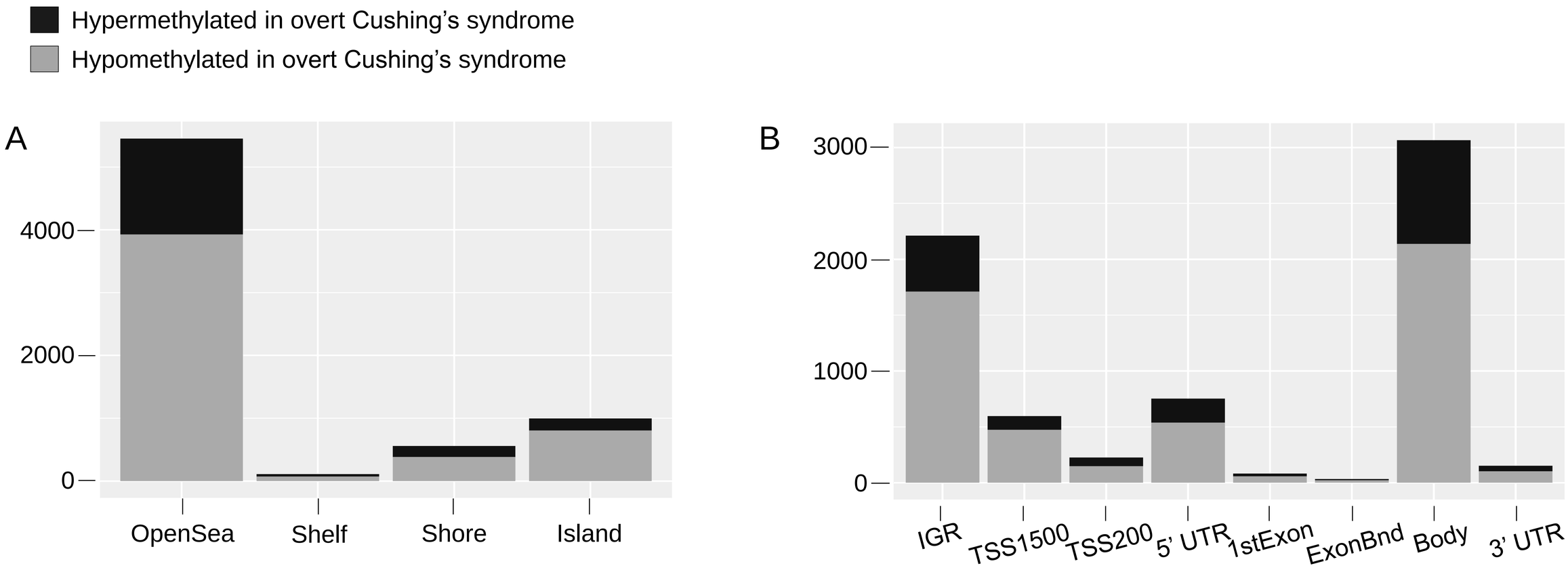
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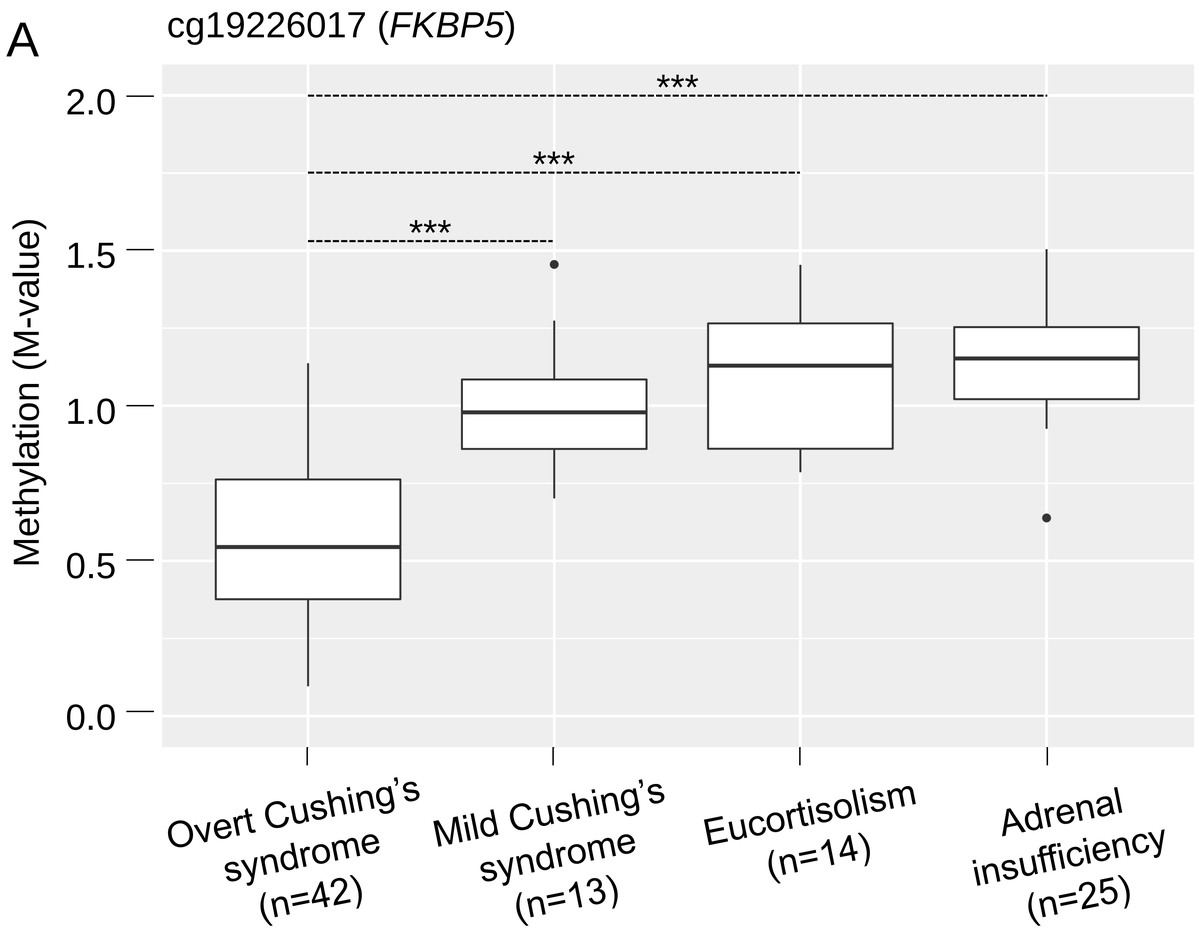
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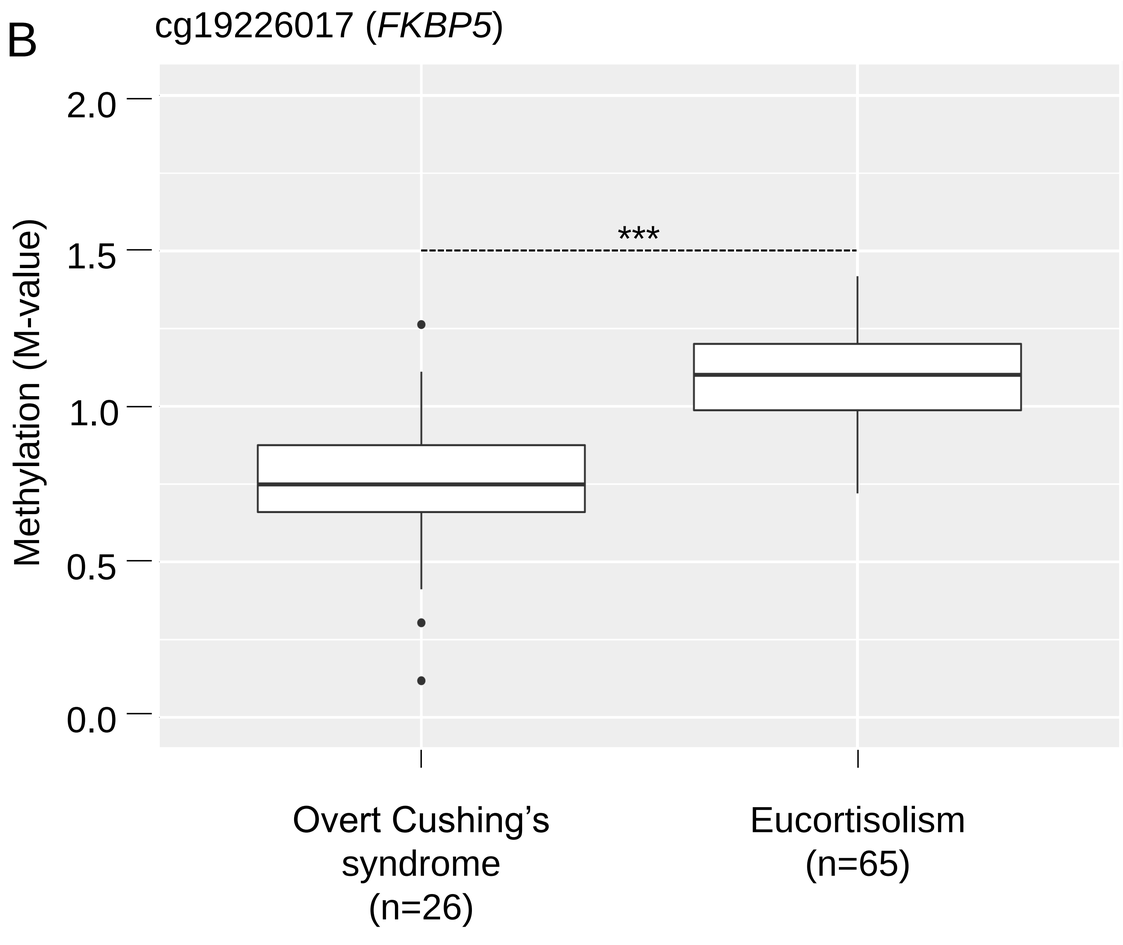


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