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Title:
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Date:
2021-05-01

Citation:
Inder, W. J., Mohamed, A., Keshvari, S., Barclay, J. L., Ruelcke, J. E., Stoll, T., Nolan, B. J., Cesana-Nigro, N. & Hill, M. M. (2021). Ex vivo glucocorticoid-induced secreted proteome approach for discovery of glucocorticoid-responsive proteins in human serum. Proteomics Clinical Applications, 15 (2-3), https://doi.org/10.1002/prca.202000078.

Persistent Link:
https://hdl.handle.net/11343/338114

Ex vivo glucocorticoid-induced secreted proteome approach for discovery of glucocorticoid-responsive proteins in human serum

Short title: Proteomics and glucocorticoid biomarkers

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/prca.202000078.

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List of abbreviations

B2M – beta-2 microglobulin

CPPED1 – calcineurin like phosphoesterase domain containing 1

DEX – dexamethasone

FAM49B – family with sequence similarity 49 member B

HIST1H1B – histone H1.5

HMGB2 – high mobility group box protein 2

HNRNPA2B1 – heterogeneous nuclear riboucleoproteins A2/B1

HPA – hypothalamic-pituitary-adrenal

LFQ – label free quantification

LYZ – lysozyme C

MAPRE2 – Microtubule-associated protein RP/EB family member 2

NCL - nucleolin

NPM1 – nucleophosmin-1

S100A4 – S100 calcium-binding protein A4

TKT – transketolase

VIM – vimentin

WARS – tryptophanyl-tRNA synthetase - cytoplasmic

Key Words: biomarker, glucocorticoid proteomics, PBMC, secretome

Word Count:

Body of text: 4572 words

Statement of Clinical Relevance:

Glucocorticoids, used in pharmacological doses for a variety of medical conditions, and Cushing's syndrome which results from hypersecretion of the endogenous glucocorticoid cortisol result in several adverse effects, including an increased mortality. Currently there is no clinically useful biomarker of glucocorticoid activity which has utility as a diagnostic test or in the field of therapeutic drug monitoring. This study has used an *ex vivo* PBMC secretome approach to discover glucocorticoid-responsive proteins, followed by clinical validation in serum of healthy volunteers

after the administration of oral dexamethasone. Using this process, we have identified three novel glucocorticoid-responsive circulating proteins as potential biomarkers of glucocorticoid activity in humans. It is clinically apparent that patients differ markedly in their therapeutic response and side effect profile when on glucocorticoids, and clinicians have no reliable measure of their biological glucocorticoid activity. Future studies will examine their utility in the diagnosis of both cortisol excess and deficiency syndromes, and in the monitoring of patients prescribed glucocorticoids.

Abstract

Purpose: To identify glucocorticoid-responsive proteins measurable in human serum that may have clinical utility in therapeutic drug monitoring and the diagnosis of cortisol excess or deficiency.

Experimental Design: A phased biomarker discovery strategy was conducted in two cohorts. Secretome from peripheral blood mononuclear cells (PBMC) isolated from 6 volunteers after *ex vivo* incubation ± dexamethasone 100 ng/mL for 4h and 24h was used for candidate discovery and qualification using untargeted proteomics and a custom multiple reaction monitoring mass spectrometry (MRM-MS) assay, respectively. For validation, five candidates were measured by immunoassay in serum from an independent cohort (n=20), sampled at 1200h before and after 4 mg oral dexamethasone.

Results: The discovery secretome proteomics data generated a shortlist of 45 candidates, with 43 measured in the final MRM-MS assay. Differential analysis revealed 16 proteins that were significant in at least one of two time points. In the validation cohort, 3/5 serum proteins were dexamethasone-responsive, two significantly decreased: lysozyme C (P<0.0001) and nucleophosmin-1 (P<0.01), while high mobility group box 2 significantly increased (P<0.01).

Conclusions and clinical relevance: Using an *ex vivo* proteomic approach in PBMC, we have identified circulating glucocorticoid-responsive proteins which may have potential as serum biomarkers of glucocorticoid activity.

1. Introduction

Glucocorticoids are frequently prescribed for a diverse range of medical conditions, with estimates that 1% of the general population are long term users.[1] They are indicated in the treatment of various inflammatory and autoimmune conditions, for immunosuppression and in physiological doses for primary and secondary adrenal insufficiency. Glucocorticoids in excessive dose can result in significant adverse effects including weight gain, hypertension, abnormalities in glucose metabolism, sarcopenia, osteoporotic fracture and skin changes.[2] They also result in suppression of endogenous hypothalamic-pituitary-adrenal (HPA) axis function, such that plans to cease glucocorticoids must be accomplished via a slow wean to allow recovery.[3] Furthermore, several studies have now confirmed an independent association between prednisolone dose used in common medical conditions, and all-cause mortality.[4, 5] The individual response to glucocorticoids is clinically variable, both in terms of therapeutic benefit and frequency/severity of adverse effects. A glucocorticoid-response biomarker, readily measurable in peripheral blood samples might enable the clinician to optimize the glucocorticoid dose, similar to how diabetes medications are titrated to glycated haemoglobin.

Our group recently reported that a ratio of an upregulated protein, thrombospondin-1, to a down regulated protein, osteocalcin can indeed reflect the total body glucocorticoid activity in humans

with endogenous and exogenous glucocorticoid excess.[6] The aim of this study was to explore the possibility of additional circulating glucocorticoid responsive proteins being measurable in humans.

Serum proteomic biomarker discovery often utilises a depletion strategy to remove high abundant serum proteins that could mask lower abundance protein quantification. While the depletion strategy has been successful, there are several disadvantages including uncharacterised loss of non-target proteins due to protein-protein binding, additional processing steps that increases the variability of data, and high cost for depletion reagents. To overcome this technical challenge, we adopted an *ex vivo* secretome proteomics strategy to identify glucocorticoid-response biomarkers, based on the assumption that proteins released from peripheral blood mononuclear cells (PBMC) in response to glucocorticoid treatment *ex vivo* will be good indicators of glucocorticoid response. PBMCs comprise of diverse innate and adaptive immune cells including lymphocytes (T, B and, Natural Killer cells), monocytes, macrophages and neutrophils. As glucocorticoids can act on almost all peripheral immune cell types[7], we hypothesized that identification of glucocorticoid-responsive secreted proteins on PBMCs *ex vivo* would yield potential biomarkers whose serum concentration would change significantly after glucocorticoid exposure in humans.

Here, we report the discovery of candidate biomarkers using *ex vivo* stimulated PBMC secretome profiling, followed by independent clinical cohort validation using serum immunoassay.

2. Materials and Methods

2.1 Experimental design

This comprised of a secretome proteomics phase (n=6 healthy volunteers) for biomarker candidate discovery and qualification, followed by an independent validation phase (n=20 healthy volunteers). As depicted in **Figure 1**, samples (PBMC or serum) from the same donor were used as baseline for each measurement.

2.2 Participant recruitment

Participants provided written informed consent and the study was approved by the Metro South Human Research Ethics Committee (HREC/14/QPAH/24). For the discovery phase, venous blood from 6 healthy volunteers (3 male, 3 female) was collected for PBMC. The validation phase involved 20 independent healthy volunteers, 10 male and 10 female, mean age 28 years (range 21-50 years). The healthy volunteers were recruited by advertisement. Exclusion criteria included age <18 or >65 years and any condition (including medication use) known to affect the HPA axis.

2.3 Ex vivo PBMC secretome

For PBMC preparation, 10 mL of blood collected in EDTA was gently layered under 7 mL of Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and centrifuged at 500 g for 20 min without brake. The interphase containing PBMCs was transferred to a fresh tube and cells were washed twice in 50 mL PBS, then resuspended in 25 mL PBS and spun at 200 g for 10 min to remove platelets. PBMCs were counted and aliquots of $1x10^6$ PBMCs in Dulbecco's Modified Eagle Medium + 10% Foetal Bovine Serum were exposed to 100 ng/mL DEX or media only for 4 and 24 hours at 37°C. After

removing the treatment media, cells were incubated with serum-free and DEX-free media for 3 hours to collect the secretomes, which were concentrated and protein concentration estimated by Bradford assay as previously described.[8]

2.4 Untargeted proteomics

Detailed proteomics methods are provided in Supplementary Methods. Secretome proteins (20 µg) were trypsin-digested by Filter Aided Sample Preparation method[9] using Microcon YM-30 (Merck-Millipore) with addition of 0.02 µg chicken ovalbumin. Tryptic peptides were desalted using OMIX C18 tips (Agilent, Santa Clara, CA). Peptides were dried, re-suspended in 20 µL of 0.5% TFA, then 1 µL was analyzed on a Q-Exactive Plus Orbitrap MS with Easy-nLC 1000 (Thermo Scientific), EASY-Spray PepMap RSLC C18 2 µm column (25 cm x 75 µm ID) with a NanoViper Acclaim C18 guard (2 cm x 75 µm).

Data were analysed using MaxQuant (version 1.5.8.3)[10] with Andromeda[11] against SwissProt (Homo sapiens reference proteome ID UP000005640, June 2017) and contaminant database (MaxQuant; 247 entries). False discovery rates (FDRs) were 1% for peptide and protein levels. Label-free quantification (LFQ) intensities were log2 transformed. Proteins with <2 unique peptides or <50% of valid LFQ values across all 24 samples were removed. Imputation was performed by replacing missing LFQ values from normal distribution. Histograms before and after imputation confirmed the overall ratio distribution is similar. Differential expression analysis was done by paired two-sample *t*-test of LFQ, comparing between DEX and its respective control in Perseus (version 1.5.8.5) [12] and R[13] Volcano plots were plotted using ggplot2.[14]

2.5 Targeted proteomics

A scheduled multiple reaction monitoring (MRM) method measuring 179 peptides from 44 proteins (43 biomarker candidates plus internal standards, chicken ovalbumin and iRT peptides) was developed on a standard-flow Nexera X2 UHPLC coupled to a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu) as detailed in Supplementary Methods. Internal standard protein (225 ng ovalbumin) was spiked into each secretome sample, and then processed by trypsin co-precipitation.[15] Individual patient MRM data was analysed in Skyline-daily as outlined previously.[16] Peak picking was manually inspected, and peak area for each transition was exported to R. Top *n* transitions that collectively account for at least 50% of the total intensity were summarized to protein intensities. Non-correlated peptides within each protein (Pearson's correlation <0.6) were removed. Peptide intensities were normalized within each protein using the formula:

$$NormalizedIntensity = SampleIntensity * AverageProteinIntensity / AveragePeptideIntensity$$

The average normalized peptide intensities were summarized to obtain protein-level intensities, which were log2-transformed prior statistical testing. Differential expression analysis was done by paired two-sample *t*-test using R.[13]

2.6 Serum validation by immunoassay

The following immunoassay enzyme linked immunosorbent assays (ELISA) were performed as per manufacturer instructions: β2 microglobulin (B2M, R&D Systems, MN, USA), lysozyme C (LYZ, Aviva Systems Biology, CA, USA), high mobility group box protein 2 (HMGB2, LSBio, WA, USA), nucleophosmin-1 (NPM1, LSBio, WA, USA) and nucleolin (NCL, LSBio, WA, USA). B2M sensitivity was

0.132 mg/L with intra assay precision coefficient of variation (CV) of <7.5% and inter-assay precision CV of <18.4%. LYZ sensitivity was 1.1 ng/mL with intra assay precision CV of <4.8% and inter-assay precision CV of <8.2%. HMGB2 sensitivity was 310 pg/mL with intra assay precision CV of <4.6% and inter-assay precision CV of <7.1%. NPM1 sensitivity was 0.115 ng/mL with intra assay precision CV of <10% and inter-assay precision CV of <12%. NCL sensitivity was 60 pg/mL with intra assay precision CV of <10% and inter-assay precision CV of <12%. Statistical analysis on the change in serum concentration of each protein following DEX was undertaken using the paired Student t test except in the case of NPM1 where the data failed to satisfy parametric assumptions and was therefore analysed using the Wilcoxon signed rank test. Normally distributed data are presented as mean ± standard error of the mean (SEM) and NPM1 is presented as median (interquartile range – IQR).

3. Results

To discover glucocorticoid-responsive serum proteins, we examined proteins released by *ex vivo* PBMCs into conditioned media following acute (4 hour) and chronic (24 hour) treatment with 100 ng/ml of DEX, each compared with respective media only *ex vivo* incubation of the same duration (**Figure 1**). Inclusion of the media treatment control turned out to be critical, as the secretome protein recovery was approximately 4-fold higher from the same number of PBMCs after 24h incubation compared to 4h. Despite starting with equal amount of total secretome protein for untargeted proteomics, we obtained twice the number of protein identifications in the 4h compared to 24h secretome, with 576±55 proteins and 280±33 proteins identified, respectively. The data completeness plot in **Figure 2A** shows that half of the samples detected considerably more proteins. Furthermore, the principal components analysis (PCA) plot in **Figure 2B** indicate that duration of treatment was the major factor in proteome divergence. These results further emphasize the importance of the media-only treatment controls and support our analytical approach to compare the fold change at each of the timepoints.

Therefore, to select promising DEX-responsive proteins for qualification, we conducted matched pairwise comparisons between the DEX+ and DEX– conditions of each time point. For this discovery analysis, p-value adjustment was not applied as the goal was to select promising candidates. In total, 45 proteins were significantly different at $P<0.05$ (**Figure 2C)**. Only 2 differential proteins overlapped between the 2 time points, namely, Transketolase (TKT) and Microtubule-associated protein RP/EB family member 2 (MAPRE2). To further evaluate the secretome differences between acute and chronic DEX treatment, a pairwise correlation heatmap was generated for 4h vs 24h data (**Figure 2D**). This showed a strong positive correlation between the majority of proteins, but a strong negative correlation for a small cluster of 15 genes of mostly cytoskeletal proteins, which were only detected at 24h (**Figure 2D**).

3.1 Qualification of candidate biomarkers

Due to the small fold change and the characteristic level of missing data points in untargeted proteomics datasets (9% and 12% of data points were "missing" from 4 and 24h, respectively), we next quantified selected candidates using custom scheduled multiple reaction monitoring method in the secretome samples. Two candidate proteins were not included in the assay due to a lack of unique peptides. After quality filtering, the MRM dataset generated protein level intensity data for 39 biomarker candidates (**Figure 3A)**. Differential analysis revealed 16 proteins that were significant in at least one of the two time points (**Figure 3B**). Notably, all 14 proteins significant at 4h were down-regulated, 11 of which were up-regulated at 24h (**Figure 3B, C**). Many candidate markers showed opposite directionality between acute and prolonged stimulation. Although less than 50%

concordance was observed across all data points, 14 out of 16 markers were consistent for at least 1 time point between the two proteomics methods.

Examination of the annotated functions and cellular components for the 16 validated DEX-responsive proteins using UniProtKB revealed typical immune cell responsive pathways and "Extracellular region or secreted" (**Table 1**). Interestingly, most of the qualified proteins were also annotated with "extracellular vesicle", and all had previously been reported in extracellular vesicle proteomics experiments as captured by Vesiclepedia.[17] Indeed, all proteins detected showed strong enrichment for GO Cellular component extracellular exosome (*P*<10e-180) and GO Biological process vesicle mediated transport (*P*<10e-55).

3.2 Validation of qualified biomarkers

Five proteins with the greatest fold-change at 24h were selected for serum validation in an independent cohort of 20 healthy volunteers: B2M, LYZ, HMGB2, NPM1 and NCL. Serum was collected at baseline (1200h) and 12h after taking a single dose of 4 mg DEX at midnight. This time was chosen as it corresponded to the maximum effect of DEX on circulating thrombospondin-1.[18] Both pre- and post-DEX blood samples were drawn at 1200h to eliminate diurnal changes in concentration as a cause for any observed differences.

LYZ and NPM1 significantly decreased following DEX: LYZ – 101 ± 5.5 vs 67 ± 4.4 ng/mL, (*P*<0.0001, **Figure 4A**); NPM1 – median 16.6 (IQR 14.4-18.4) vs 14.2 (IQR 11.1-17.4) ng/mL, (*P*<0.01, **Figure 4B**), while HMGB2 significantly increased – 819 ± 34 vs 984 ± 60 pg/mL (*P*<0.01, **Figure 4C**). There was no

significant change in the concentration of B2M (0.442 ± 0.034 vs 0.463 ± 0.051 mg/L, *P*=0.71, **Figure 4D**), and NCL was below the detection limit of the assay in serum (data not shown). Next, we examined responses at an individual level, and found that LYZ decreased in 100%, NPM1 decreased in 80%, while HMG2 increased in 75% of participants.

As we previously introduced the concept of a ratio between an up- and down-regulated serum proteins,[6] we also investigated ratios between HMGB2 and LYZ. Encouragingly, we found that the ratio between HMGB2 and LYZ increased in 90% of participants following DEX (from 8.4 ± 0.45 to 15.7 ± 1.14, *P*<0.0001), showing potential utility as a clinical indicator of DEX responsiveness.

4. Discussion

The need for biomarkers of glucocorticoid activity has been highlighted in recent reviews including in the assessment of adequacy of replacement in adrenal insufficiency [19] and in the pharmacological treatment of asthma.[20] This study demonstrates the successful application of *ex vivo* PBMC secretome to identify glucocorticoid responsive serum proteins, three of which were subsequently validated in the serum concentrations of healthy volunteers who took a single dose of oral DEX. These new glucocorticoid response serum protein biomarkers warrant further validation in additional clinical cohorts. While the glucocorticoid-responsive proteins we have identified are unlikely to change in concentration solely in response to alterations in glucocorticoid activity, by combining biomarkers in a panel, the specificity is likely to increase, as we have previously shown in applying a ratio between thrombospondin-1 and osteocalcin.[6]

Several innovative proteomics approaches were applied in this study. To avoid the challenges of serum proteome analytics and the caveats of abundant protein depletion or selective enrichment strategies, we pursued an *ex vivo*-stimulated PBMC secretome approach to discover potential acute and chronic glucocorticoid-responsive proteins. The cellular secretome was collected over 3 h, after 4h or 24h *ex vivo* culture ±DEX. While the 24h secretome returned 4-fold more total protein amount compared to 4h, it returned only half the protein identifications by proteomics. This effect was not influenced by glucocorticoid treatment and may be due to the deterioration of PBMC during prolonged *ex vivo* culture, leading to spillage of partially degraded cellular proteins. Importantly, our experimental design had accounted for these potential differences by including a media-only control for both *ex vivo* time points, and conducting pairwise comparison for each donor. These measures allowed the successful discovery and validation of novel glucocorticoid response biomarker candidates (LYZ, HMGB2) from the 24h treatment condition.

LYZ (Lysozyme C, conventional) is a crucial component of the innate immunity against bacterial infection, with both enzymatic and non-enzymatic activity.[21] Only a single report previously associated LYZ with DEX action, on a differentiated monocytic cell line.[22] To our knowledge, this is the first study to show that acute exogenous glucocorticoid exposure *in vivo* in humans reduced circulating serum LYZ. As serum LYZ was decreased 12h following DEX in all 20 participants, it is a particularly promising candidate as a potential circulating biomarker of glucocorticoid response.

HMGB2 (previously called HMG2) is a member of the high-mobility group (HMG) proteins that are multifunctional proteins with a canonical role as chromatin-associated proteins in the nucleus. HMG proteins promote transcriptional regulation of steroid receptors including glucocorticoid receptors

by promoting their DNA binding activity.[23] While HMGB1 and HMGB2 have the ability to enhance GR mediated transcriptional activation in a DEX-responsive manner when overexpressed in cell culture,[23] a role for HMG proteins in glucocorticoid action has not been investigated *in vivo*. An increase in plasma HMGB2 has been observed during ageing and functional decline, with *in vitro* evidence suggesting HMGB2 is released from senescent endothelial cells.[24] Circulating HMGB2 is also increased during following myocardial infarction and is independently associated with major adverse cardiac events.[25] It is noteworthy that there is also an age-related increase in serum cortisol[26] and following myocardial infarction[27] in parallel to that of HMGB2, although there is no current evidence of a causal relationship between the higher cortisol (the main endogenous glucocorticoid) and HMGB2. Further research is required to determine if the increase in serum HMGB2 observed in this study after DEX in healthy volunteers relates specifically to glucocorticoid stimulation, in which case we would hypothesise elevated levels in patients with Cushing's syndrome and on chronic prednisolone therapy.

NPM1 is also a multifunctional protein with roles in chromatin remodelling, genome stability, mRNA transport and ribosome biogenesis.[28] While NPM1 has not been directly linked with glucocorticoid activity or GR receptor, an interesting parallel with HMGB2 is the critical role of nuclear-cytoplasmic regulation of NPM1 in cellular health. NPM1 is overexpressed or mutated in a variety of cancers including gastric, prostate and colon cancer, where it aberrantly locates in the cytoplasm. NPM1 mutations are found in approximately one third of cases of acute myeloid leukemia (AML), including 50-60% of AML cases with a normal karyotype.[28] Interestingly, DEX has greater anti-leukemic activity in AML with NPM1 mutations, hinting at a functional link.[29]

An intriguing result from the study is the inference of extracellular vesicle (EV) in glucocorticoid action. The three confirmed candidates were previously reported in diverse EVs by immuno-electron microscopy (LYZ),[30] LC-MS/MS of neutrophil EVs (HMGB2),[31] malignant B cells,[32, 33] U937-differentialed macrophage[34] and apoptotic Jurkat T-cells (NPM1).[35] As immune cell-derived EVs are immuno-modulatory,[36] the potential of glucocorticoid-induced EV release should be investigated.

In summary, this study provides evidence of three novel circulating glucocorticoid responsive proteins, identified using a proteomic approach. Patients treated chronically with a given dose of prednisolone can have a broad range in clinical severity of glucocorticoid-related adverse effects, and patients with Cushing's syndrome also vary considerably in phenotype. Therefore, is it apparent that the biological effect of glucocorticoids is not simply dose dependent. Having a readily measurable serum biomarker or panel of biomarkers may allow the clinician to optimize the glucocorticoid dose for an individual patient, providing improved therapeutic efficacy and/or reduced side effects. Glucocorticoid biomarkers may also assist in distinguishing mild Cushing's syndrome from pseudo-Cushing's states, a clinical scenario which remains very challenging. Further study is ongoing to determine if these three proteins have clinical utility as biomarkers of glucocorticoid activity in humans.

5. Associated Data

Proteomics data are available via PRIDE [37] data set identifier PXD019945 [Reviewer username: reviewer29367@ebi.ac.uk and Password: ntI8FU3H].

Skyline files of MRM experiments are published on PanoramaWeb[38] (https://panoramaweb.org/Hill_glucocorticoid_response.url) and raw data deposited to PanoramaWeb[39] (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD019972) [Email: panorama+qimr5@proteinms.net and Password: AMcFw90t].

Acknowledgements

This research was funded by a grant from the Princess Alexandra Research Foundation. We thank the Translational Research Institute Proteomics Core Facility and the University of Queensland Centre for Clinical Research Mass Spectrometry Facility for technical support. The Translational Research Institute is supported by a grant from the Australian Government.

Conflict of Interest Declaration

The authors have no conflicts of interest to declare.

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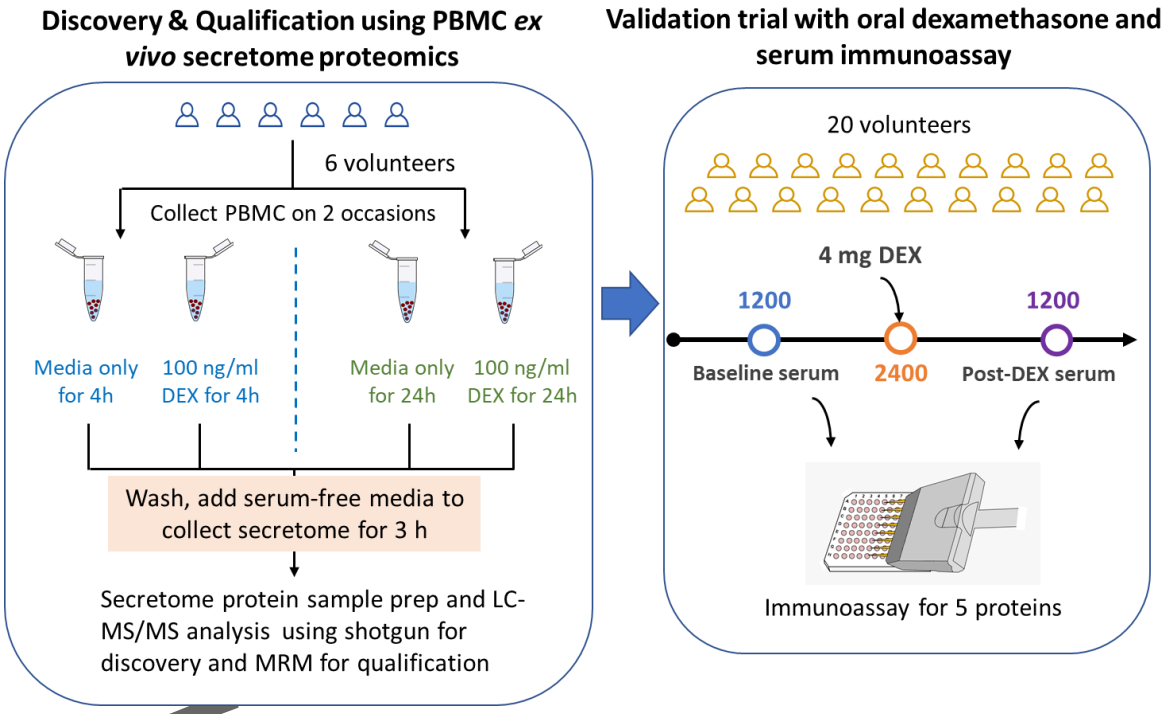

Figure 1.

Workflow for discovery and validation of glucocorticoid-response proteins in human serum.

Candidate glucocorticoid-response proteins were discovered through *ex vivo* peripheral blood mononuclear cell (PBMC) secretome profiling following dexamethasone (DEX) or buffer treatment for 4 or 24 hours. For each time point and donor PBMC, paired analysis was conducted to generate a list of candidate DEX-responsive proteins. A custom multiple reaction monitoring (MRM) assay was developed for quantitative proteomics of the same secretome samples. Serum levels of 5 candidate proteins were measured by immunoassay in an independent validation trial, where serum was collected from 20 volunteers before and after oral DEX.

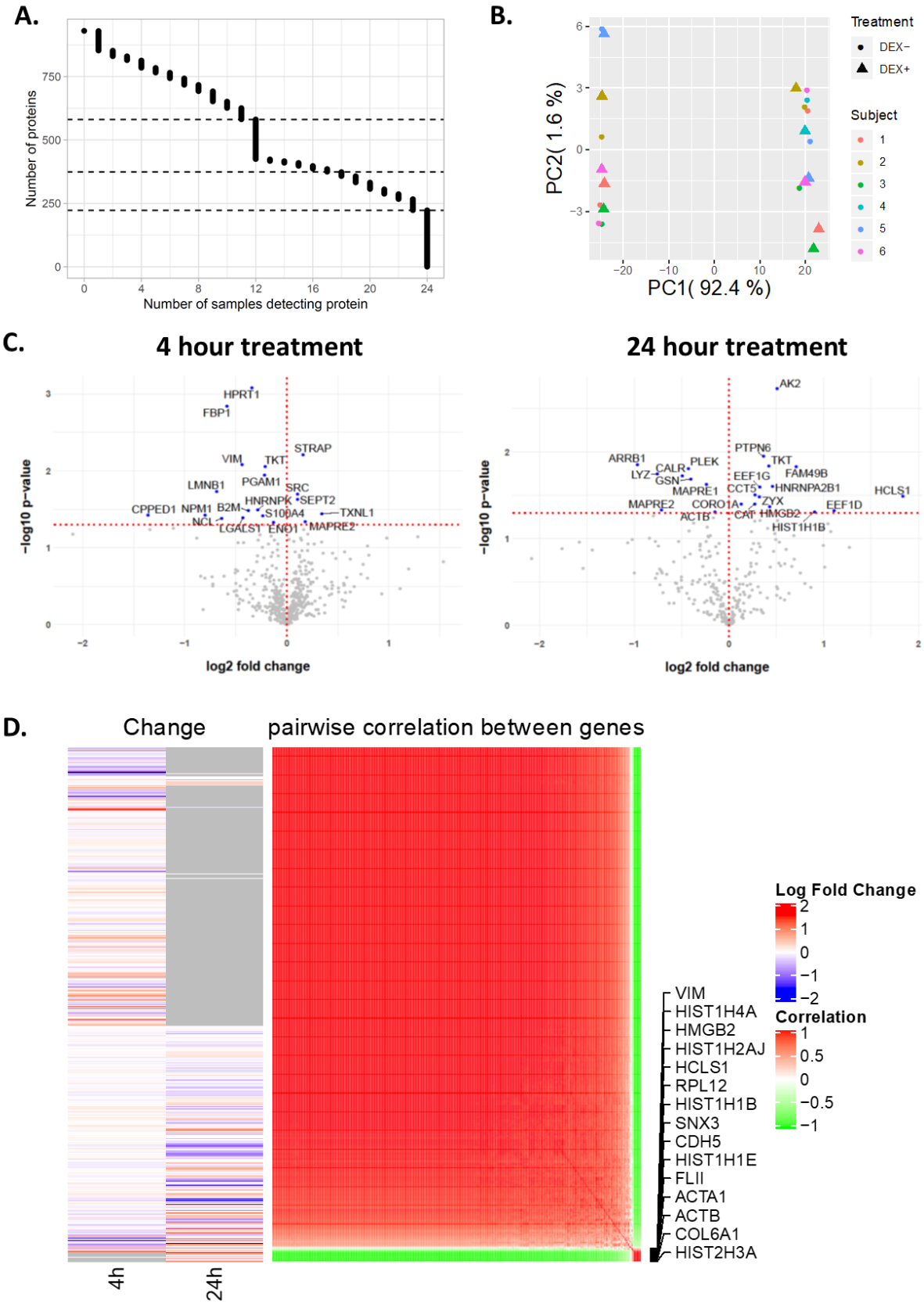

Figure 2.

***Ex vivo* secretome profiling data.** Secretome samples as collected in Figure 1 were analysed by LC-MS/MS.

A. Data completeness plot showing the number of proteins detected in the number of samples. Lines represent 50%, 75% and 100% detection rate (581, 386 and 223 proteins respectively).

B. Principal component analysis with each paired secretome sample marked by subject and duration of *ex vivo* treatment.

C. Volcano plots showing log2 fold change (x-axis) and –log10 p-value (uncorrected, y-axis) of proteins detected in the secretome of PBMCs following 4h and 24h 100 ng/mL DEX treatment, compared to control secretome which was treated with media for 4 hours. N=6. The horizontal dotted line indicates *P*=0.05.

D. Overview of protein correlation and change in profiling data. Protein pairwise correlation heatmap (left) and their corresponding log fold change (left) showing 2 distinct negatively-correlating clusters. A small cluster of 15 genes (mostly cytoskeletal) were only present after 24hr of DEX treatment. Overrepresentation analysis of the protein cluster using G:Profiler[40] showing significant enrichment of protein-containing complex binding and structural constituent of cytoskeleton gene sets. Grey bars indicate not detected.

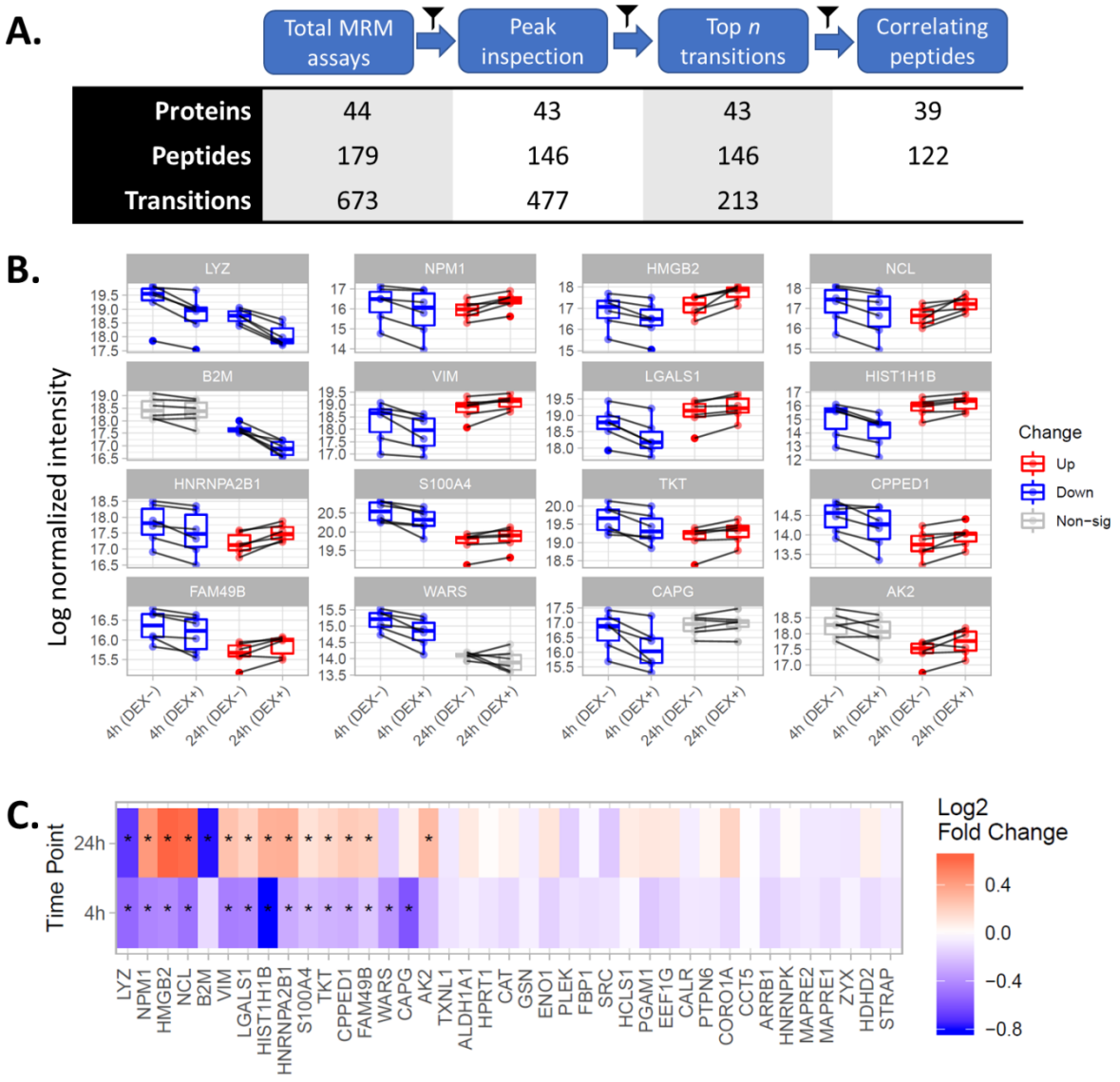

Figure 3.

Qualification of secretome biomarker candidates using targeted proteomics. *Ex vivo* secretome samples were analysed by multiple reaction monitoring (MRM) assay designed against the selected candidate biomarkers. A. MRM data analysis pipeline and resulting proteins/peptides passing each processing step. B. Fold change of secretome biomarker candidates for 4h and 24 h DEX treatment. * $P<0.05$. C. Comparison of direction of change for the 16 qualified biomarkers between the MRM data (Validation) and Discovery proteomics data.

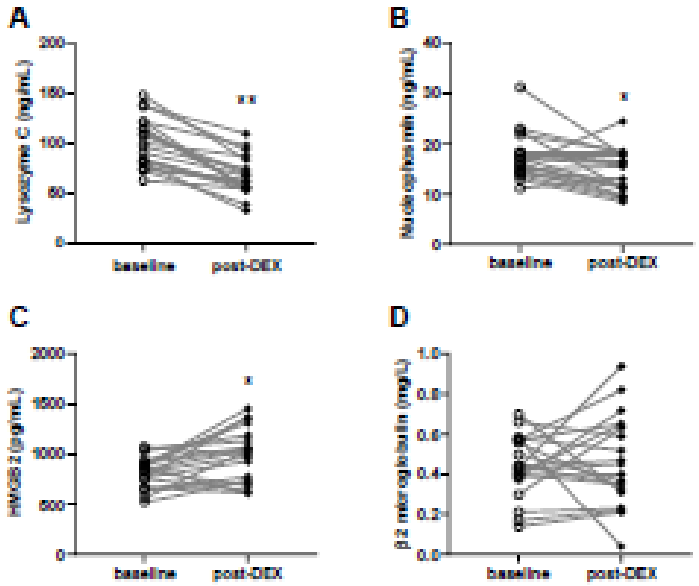

Figure 4.

Immunoassay of candidate serum biomarkers. Validation of selected candidate biomarker as depicted in Figure 1. Immunoassays results as shown for the four detectable proteins.

A. Lysozyme C. B. Nucleophosmin. C. High mobility group box 2 (HMGB2). D. β2 microglobulin. * *P*<0.01. ** *P*<0.001.

Table 1. Functions and cellular components of proteins altered in the ex vivo PBMC secretome qualified by targeted proteomics.

Protein	Gene names	UniProt	Biological Pathway (Reactome)	Cellular compartments
Lysozyme C	LYZ	P61626	Neutrophil degranulation	Extracellular region or secreted, Lysosome, Other locations
Nucleophosmin	NPM1	P06748	Nuclear import of Rev protein SUMOylation of transcription cofactors	Cytoskeleton, Cytosol, Nucleus, Other
High mobility group protein B2	HMGB2	P26583	Apoptosis induced DNA fragmentation	Extracellular region or secreted, Nucleus, Other
Nucleolin	NCL	P19338	rRNA processing in the nucleolus and cytosol	Extracellular region or secreted, Nucleus, Other
Beta-2-microglobulin	B2M	P61769	ER-Phagosome pathway Interferon gamma signaling	Cytosol, Endoplasmic reticulum, Endosome, Extracellular region or secreted, Golgi apparatus, Plasma membrane, Other

Vimentin	VIM	P08670	Chaperone Mediated Autophagy Interleukin-4 and Interleukin-13 signaling	Cytoskeleton, Cytosol, Extracellular region or secreted, Nucleus, Peroxisome, Plasma Membrane, Other
Galectin-1	LGALS1	P09382	Post-translational protein phosphorylation	Cytosol, Endoplasmic reticulum, Extracellular region or secreted, Nucleus, Other
Histone H1.5	HIST1H1B	P16401	Apoptosis induced DNA fragmentation	Nucleus, Other
Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	P22626	mRNA Splicing	Extracellular region or secreted, Nucleus, Other
Protein S100-A4	S100A4	P26447	N/A*	Extracellular region or secreted, Nucleus, Other
Transketolase	TKT	P29401	Pentose phosphate pathway	Cytosol, Extracellular region or secreted, Peroxisome, Other locations
Serine/threonine-protein phosphatase CPPED1	CPPED1	Q9BRF8	Neutrophil degranulation	Cytosol, Extracellular region or secreted, Lysosome, Plasma membrane

CYFIP-related Rac1 interactor B	FAM49B	Q9NUQ9	Platelet degranulation	Extracellular region or secreted, Mitochondrion, Other
Tryptophan--tRNA ligase	WARS1	P23381	Cytosolic tRNA aminoacylation	Cytosol, Extracellular region or secreted, Nucleus, Other
Macrophage-capping protein	CAPG	P40121	N/A*	Cytoskeleton, Extracellular region or secreted, Nucleus, Other
Adenylate kinase 2	AK2	P54819	Interconversion of nucleotide di- and triphosphates	Extracellular region or secreted, Mitochondria

* Not mapped to a Reactome pathway