Pharmacogenomics of the Variability in the *In Vivo* Response to Glucocorticoids

NCT03023891

10/23/17

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1.0 Background

Glucocorticoids (GCs) are commonly used worldwide to treat several conditions, including asthma, lymphomas, and chronic inflammatory disorders.[1,2] It is estimated that between 1-3% of the general population in developed countries have used GCs, and that 65-75% of these patients used GCs chronically (≥ 3 months).[1,3] Although GCs have dramatic anti-inflammatory and immunosuppressive effects, they exhibit **large inter-individual variability in GC response in terms of efficacy and toxicity**. This variability in GC response explains why nearly 30% of patients with different pathological conditions have a suboptimal response to the anti-inflammatory effects of GCs[4-7] resulting in morbidity and substantial economic costs.[8] Moreover, it explains why between 10 to 80% of patients who receive GCs chronically develop serious side effects.[9-11]

2.0 Rationale and Specific Aims

The large inter-individual variability in GC response has a genetic component, but the genetic architecture of GC response is still unknown. A few studies have linked a small number of genetic variants with GC therapeutic efficacy.[12-15] One reason for the lack of progress in identifying the genetic determinants of GC efficacy has been the measurement of response in clinically heterogeneous phenotypes (e.g., asthma, inflammatory bowel disease). These phenotypes require weeks to respond to GCs, and the pharmacogenetic signal is susceptible to confounding by disease heterogeneity and co-therapies. A more attractive approach is to study a "clean" intermediate phenotype — a pharmacodynamic measure of GC efficacy. GC-induced lymphopenia is such a measure; this is a rapid, reproducible, dose- and concentration-dependent response that has been quantified in human pharmacokinetic and pharmacodynamic models.[16-19] Moreover, lymphocytes are major determinants of inflammation and GCs exert part of their anti-inflammatory effects by altering lymphocyte kinetics and survival. Thus, GC-induced lymphopenia is a robust and relevant intermediate phenotype to study the genetic predictors of GC efficacy.

In addition to variability in GC efficacy, there is also marked variability in the frequency and severity of GC toxicity. One of the most common metabolic side effects of GC therapy is GC-induced glucose intolerance.[20-22] Although GC impairs glucose metabolism through insulin resistance and by β-cell dysfunction, there is large variability in the severity of the metabolic effects of GCs that is explained by clinical factors. Evidence suggest that metabolic effects of GC has a genetic component, but the genetic determinants of metabolic toxicity remains unknown.

As other complex traits, genome-wide association studies (GWAS) have identified some risk variants for GC response;[12-15] however the functional relevance for most of these variants are not known. In complex traits, the combination of GWAS information and large gene expression data has shown that top hits on GWAS were enriched for polymorphisms that regulate expression levels of genes (regulatory polymorphisms),[23] and that these regulatory variants explained a large proportion of the phenotypic variability for complex phenotypes including drug response.[24, 25]

GCs are known to exert their effect through changes in gene expression and also by interacting with several transcription factors,[26,27] but we still do not know which genes are involved in the response to *in vivo* administration GC in humans, and which genetic variants are more likely to affect the expression of these genes. Thus, we propose **to perform a hypothesis-generating pilot study to identify potential set of genes and**

regulatory variants that are likely to be involve in the response to GC in terms of efficacy and metabolic toxicity.

For this pilot study we plan to use a robust pharmacodynamic effect of GCs (GC-induced lymphopenia) and a common metabolic complication of GC therapy (GC-induced glucose intolerance) after *in vivo* administration of GC as our clinical phenotypes to:

Specific Aim: To characterize gene expression patterns that are predictive of *in vivo* GC response, and identify regulatory variants that are associated with *in vivo* GC response by challenging 40 healthy volunteers with 60 mg of oral prednisone. We will measure changes in lymphocyte counts and changes in glucose regulation (using the 75 gr glucose tolerance test ~ OGTT) as surrogates of GC efficacy and metabolic toxicity. We will perform transcriptome-wide next generation RNA sequencing to measure changes in gene expression due to *in vivo* administration of GC and study the association of gene expression patterns with our clinical phenotypes. Once we identify a set of genes that are significantly changed after *in vivo* GC administration, we will select potential regulatory variants that have been associated with changes in expression of these genes in public available expression quantitative trait loci (eQTL) datasets to perform genome interrogation and eQTL analysis. This will allow us to identify variants that are associated with gene expression changes due to *in vivo* administration of GC.

3.0 Animal Studies and Previous Human Studies

Pharmacodynamic measures of GC efficacy: One of the mechanism through which GC exerts its anti-inflammatory effects is by modifying the traffic and function of immune cells.[18,28,29] GCs markedly increase the number of circulating neutrophils and decrease the number of circulating white blood cells (WBC).[30,31] Among WBC, neutrophils and lymphocytes have been largely use to study the pharmacodynamics of GCs;[16,17,32,33] mostly because the other leukocytes represent a small percentage of the circulating WBC and the administration of GCs reduced greatly their number (sometimes to undetectable levels) making difficult to establish a dose-concentration response.[31] Not only the changes induced by GCs in circulating neutrophil and lymphocyte counts are dose- and concentration-dependent,[34] reproducible,[35] and obtained with equivalent doses of commonly used GCs;[16] but also the same response can be evoked in healthy individuals and in patients with inflammatory disorders.[34,36,37] Thus, changes in WBC counts are well-established and reproducible pharmacodynamic phenotypes to study the genetics of GC response.

Metabolic side effects of GCs – glucose intolerance: There is also large variability in GC toxicity. Glucose intolerance is a frequent and clinically relevant metabolic complication of GC therapy[21,22] that can lead to transient hyperglycemia[38] or to a permanent condition called GC-induced diabetes.[39,40] The mechanisms through which GCs induce glucose intolerance are similar to those involved in the development of type 2 diabetes mellitus (T2DM),[39,41,42] and include insulin resistance[43] and β-cell dysfunction.[44,45] Hyperglycemia occurs when β-cell failure can no longer compensate for the degree of insulin resistance. Clinical studies have shown that short-term administration of GCs impairs glucose tolerance in individuals with normal glucose tolerance;[44,46-48] while a high single doses of GCs have shown to increase insulin resistance[44,46,48,49] and consequently increase insulin secretion[44,46] a compensatory mechanism that is reduced in individuals at risk for T2DM.[44,48,50-53] Evidence suggest that

glucose intolerance during GC therapy is in part due to a genetic component since the degree of glucose intolerance varies markedly among patients who received the same dose of GCs;[54] there is large variability in the incidence of glucose intolerance due to GC therapy among races;[55,56] and variants known to affect GC sensitivity have been associated with metabolic phenotypes[57-65] Despite these observations, the genetic determinants of GC–induce metabolic toxicity are not known and we cannot predict who will develop a metabolic complication during GC therapy.

GC exert their effects through the regulation of gene expression by regulatory variants: GCs are known to exert their effect through changes in gene expression and by interacting with several transcription factors. Thus, they represent an excellent tool to study gene-environment interactions.[66] Accordingly, using immortalized and cultured human peripheral blood mononuclear cells (PBMCs), several studies have shown that GC response is associated with different patterns of transcriptional response,[14,27,67] and that variants in regulatory binding sites at GC-targeted genes may play a role in GC response variabilty.[12,14,26,27] However, the process of immortalization and the culture of cells are known to affect the gene expression response and likely differ from *in vivo* expression.[68-70] Moreover, the eQTL studies using immortalized cells have little overlap with eQTL from cultured cells.[27] To our knowledge, there is only one small study that has examine gene expression changes with *in vivo* administration with GC but have not performed eQTL analysis. Olnes explored genome transcription response after *in vivo* administration of GCs using whole transcriptome microarrays,[71] and found that peak changes in gene expression were observed 4 hours after GC administration with large variability in the gene expression changes. We propose to extend Olnes' work by studying the role that genetic variants have in the observed variability of gene expression response during *in vivo* administration of GC. Besides, we propose to study PBMCs gene expression response to a glucose challenge due to *in vivo* administration of GCs to study the genetic architecture of the metabolic complications of GC administration, which has not be done.

4.0 Inclusion/Exclusion Criteria

Inclusion criteria:

- 18 to 45 years old (to exclude the effect of age on glucose tolerance)
- BMI between 20 and 30 kg/m^2 (to exclude individuals that are likely to have impaired insulin response)
- Normal fasting glucose
- Stable weight for three months before participation

Exclusion criteria:

- BMI ≥30kg/m^2
- Fasting glucose ≥126 mg/dl
- Shift work or disordered sleep (to exclude individuals with alterations in the hypothalamus-adrenergic axis)
- Any active diseases (requiring current treatment)
- Use of any medication regularly (including over-the-counter such as aspirin, ibuprofen, etc)
- Previous exposure to GCs (within the last year)
- Diabetes
- Pregnant or breastfeeding

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5.0 Enrollment/Randomization

We plan to recruit participants by sending notices through ResearchMatch.org, Research notifications email, word of mouth, and fliers.

6.0 Study Procedures

Potential participants will meet with the study team to discuss the purpose, inclusion and exclusion criteria, procedures and risk/benefits of the study. Those who sign the consent form will come for a screening visit, and if they fulfill inclusion and exclusion criteria, they will come to Vanderbilt University Medical Center (VUMC) for two study visits in the following 30 days. The study visits will take place within a one-month window, will start early in the morning and will last approximately 6 hours. For all the study visits participants will be asked to fast overnight and avoid alcohol consumption and exercise (except walking<30 minutes) 48 hours before the visit, and be free of any illness.

- Screening visit: The participant will arrive fasting early in the morning to VUMC. In this visit, the study team will ask questions about his/her health and past medical history. The study team will perform a brief physical exam that include participant's height and weight to calculate the body mass index (BMI) and waist and hip circumference. Blood samples will be collected for safety reasons and to culture peripheral blood mononuclear cells *in vitro* with glucocorticoid. We will request a urine and stool sample for future research. Women of child-bearing potential will have a urine pregnancy test. Women who are pregnant will not be allowed to participate. The study team will provide instructions about fasting and activities before the study visits. If any of the test results on the blood collected at the screening suggests that the participant may have a condition that can alter the study (e.g., random glucose>200 mg/dL) or put him/her at risk, the study team will contact the participant to explain the reason why he/she cannot participate in the study.
- Study visit 1: The participant will arrive fasting early in the morning to VUMC. A standardized clinical interview and brief physical examination will be performed. For female participants, a urine sample will be collected for pregnancy testing. A fingerstick will be perform to check fasting glucose level. An intravenous catheter will be placed in one arm for continuous sampling around 31/2 hours after the participant arrival. Once venous access is secure, blood samples will be collected at different times. If the fasting glucose is ≥126 mg/dl, the participant will be excluded from the oral glucose tolerance test study, and blood will be collected approximately 4 and 6 hours after the fingerstick. If blood glucose is below 126 mg/dl; we will collect blood samples around 4 hours after the patient's arrival. Immediately afterwards, a 75-gram glucose solution will be administered as part of the oral glucose tolerance test (OGTT), and blood samples will be collected at 10, 20, 30, 60, 90, and 120 minutes after glucose ingestion. Participants with 2-hour blood glucose ≥200mg/dl will be excluded. A standard meal will be provided at the end of the study visit.
- Study visit 2: Visit 2 will occur within one month after visit 1. An interview, physical exam, and urine testing in the fasting participant will be performed, as in Study visit 1. A single dose of 60 mg of oral prednisone will be administered immediately after a fingerstick for blood glucose is performed. Approximately 31/2 hours later, an intravenous catheter will be placed in one arm for continuous sampling. The first blood

collection will be perform around 4 hours after the ingestion of prednisone, and immediately after an OGTT will be performed (if tolerated on study visit 1). Blood collection will occur at 10, 20, 30, 60, 90, and 120 minutes after glucose ingestion. A standard meal will be provided at the end of the study visit.

Each visit will take approximately 6 hours and the participant should stay at VUMC during the study visits. The study team will contact the participant by phone for the next two consecutive days after the last visit to inquire about any problem related to the study.

Measurements: Blood samples will be used for the following:

- Basic metabolic screening
- Glucose, insulin, and C-peptide to study insulin sensitivity and β cell function[72, 73] at both study visits.
- Blood cell counts.
- RNA extraction will be performed from PBMCs collected in both study visits.
- Genotyping
- Urine and stool samples will be stored for future research. Female participants will have a urine pregnancy test at each study visit.

RNA sequence and Genotyping: RNA will be extracted from PBMC lysates following standard procedures and send to the Vanderbilt Technologies for Advance Genomics (VANTAGE) Core for RNA-seq. VANTAGE will perform RNA sequencing including small RNAs. Once sequencing data is available, VANGARD will align the reads to a reference genome, count the reads, compared gene expression levels before and after GC exposure and obtain a list of genes that are differential expressed. Genotyping will be performed at the VANTAGE Core using Megachip

7.0 Risks

Drawing of venous blood: Puncturing of a vein may result in minor bleeding or bruising. Because some people may faint during blood draw, we will ask the participants to lie down during blood collection.
Fasting: Dizziness, light-headedness, or nausea can occur as result of fasting.
75gr OGTT: nausea, vomiting, or light-headedness may occur after drinking the glucose solution. A drop in blood glucose levels may occur during the test which may cause nausea, dizziness, sweating, weakness, or restlessness. A significant drop in glucose levels during the test is likely to occur during illnesses, alcohol consumption, dieting, in patients using medications or long periods of bed rest (hospitalized patients), but rarely in healthy people. The study team will monitor the blood glucose several times during the test using a glucometer. If blood glucose levels fall out of safe ranges, the test will be stopped and oral (or intravenous) glucose solutions will be administered if needed. Transient hyperglycemia during OGTT is likely to occur in people with undiagnosed diabetes, and it resolves spontaneously once the test is complete.
Prednisone is a FDA-approved drug for several disorders. Glucocorticoids have several side effects (e.g., increase in blood pressure, electrolytes abnormalities, adrenocortical suppression, risk of infection, mood and behavioral disturbance, gastrointestinal problems, etc.), which are more likely to occur with repeated and high doses. We plan to administer a single dose of prednisone 60 mg to healthy individuals and monitor them for the next 6-6½ hours after the administration of the drug. It is unlikely that any harmful side effects will occur with a single exposure to

glucocorticoids. Prednisone has a half-life of 18-36 hours; thus, we will instruct all the participants to contact us if they experience any discomfort within the next 72 hours after the study conclusion.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

The proposed study is low risk. These studies do not fall into the traditional Phase I, II, or III clinical trial concepts. Nevertheless it is important to monitor the data obtained and subject safety. Monitoring should be commensurate with the risks, which are small. The principal investigator proposes to review the data weekly and adverse events if they occur. During the conduct of the study, any serious adverse event will be reported to the IRB within 7 days of the PI's notification of the event. Should the risk-benefit ratio of the study change the principal investigator will make alterations to the procedures of the study and submit them to the IRB.

9.0 Study Withdrawal/Discontinuation

Withdrawal of participants from any part or the whole study is under the discretion of the PI and will be done if:

- Performing a specific study procedure may be harmful for the participant or he/she cannot tolerate the procedure.
- The participant does not meet the study requirements
- The study is stopped
- Participant is not willing to continue the study

Participants who may not tolerate the OGTT may continue in the study to assess GC efficacy but not GC metabolic toxicity. The study team will explain to the participant why he/she is taken out from specific procedures or from the whole study.

10.0 Statistical Considerations

Although this is a pilot study that will provide preliminary information of the effect size and variability of our outcomes (clinical and gene expression changes), we have 85% power to detect a minimum fold change of 2.5 for a total number of 20,000 tested genes with the top 100 being prognostic with a FDR of 0.1 using exact test, assuming a minimum average read counts of 10 for prognostic genes, a maximum dispersion of 0.5, and a ratio of the geometric mean of normalization factor of 1. The main analysis of the gene expression data will be carried out by Dr. Yan Guo (VANGARD), who will perform the following procedures:

• Detect genes that on average change expression levels due to GC treatment: we will normalized gene expression data. We will use the $\log_2$ fold change in expression as a measure of response, and perform multiple linear regression controlling for potential covariates (batch, age, and BMI) with a FDR < 0.1.

• Identify a set of genes with expression patterns are predictive of GC response: to test the association between changes in our clinical outcomes (lymphocyte counts and metabolic indexes) and $\log_2$ fold change for each gene, we will perform linear regression analysis using a FDR of 0.1. We will stratify our analysis by sex. To control for the effect of estrogens levels in GC response among females, we will register the day of the menstrual cycle that the study visit occurred and include it as a covariate. For women

who are using a hormonal birth control method, we will also include the type of hormone birth control method (systemic or local), and active ingredient (progesterone with and without estrogen) as covariates.

• Select a list of candidate of regulatory variants that are likely to explain the observed variability in gene expression patterns: using public available eQTL datasets (GTEx, seeQTL, NCBI eQTL Browser, Genevar, SCAN, ExSNP, etc), we will query genes that their gene expression profile are predictive of GC response to identify a candidate list of variants for genome interrogation. We will select variants that: (a) have shown to be significantly associated with the expression of the respective gene ($P<5x10^{-4}$), (b) are located within 1 megabase on either site of the gene's transcriptional start site, and (c) have MAF >0.10. We will combined genome information and the gene expression data using a Bayes factor calculation[27] to identify which variants do affect gene expression profile with GC administration with and without OGTT.

Additionally, to determine which mechanisms are shared between in *in vivo* and *in vitro* GC exposure, we plan to culture PBMCs with GC in normoglycemic and hyperglycemic media. PBMCs will be isolate from blood samples collected at screening visit and culture for 4 hours with GC in a normoglycemic and hyperglycemic media to simulate the effect of OGTT and measure changes in gene expression. By comparing gene expression profiles from *in vivo* and *in vitro* administration of GC on PBMCs from the same individual, we can identify which mechanisms are shared in both conditions. This will largely improve our ability to perform clinical studies in the future that involve GC exposure by reducing the risk and costs related to drug exposure for specific mechanisms that are shared in *in vitro* and *in vivo* response.

11.0 Privacy/Confidentiality Issues

Confidentiality and ethical considerations will be addressed as follows. All identifying documents, data, and specimens collected as a result of this study will be retained by the investigator. The information collected will be stored in a secure, password-protected database. All samples collected will be labeled with a unique code. Access to this material will be available only to the research investigator and/or her research staff. If results of this study are to be published, only code numbers will be used for identification purposes. Participants will not be identified by name.

12.0 Follow-up and Record Retention

For this pilot study, we expect to finish participant enrollment within 2 years. However, we expect that the sequencing process, genome interrogation, and data analysis will be completed within 3 years. Research data that results from this study will be archive for unknown length of time.

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