Cortisol

Cortisol Quantification Investigation: Prospective, Observational Study Comparing Free versus Total Serum Cortisol in PICU Patients

CPCCRN Protocol Number 012

Collaborative Pediatric Critical Care Research Network
National Institute for Child Health and Human
Development (NICHD)

Version 1.04
Version Date: March 31, 2008
Printing Date: March 31, 2008

This protocol is CPCCRN Protocol Number 012, and the lead CPCCRN investigator for this protocol is Jerry J. Zimmerman, M.D., Ph.D., University of Washington, Seattle Children's Hospital.

PROTOCOL TITLE:

Cortisol Quantification Investigation: Prospective, Observational Study
Comparing Free versus Total Serum Cortisol in PICU Patients

Short Title: The CQI Study
CPCCRN Protocol Number: 012

Lead Investigator and Author:
Jerry J. Zimmerman, M.D., Ph.D.
University of Washington
Seattle Children's Hospital

Protocol Version: 1.04
Version Date: March 31, 2008

I confirm that I have read this protocol, I understand it, and I will conduct the study according to the protocol. I will also work consistently with the ethical principles that have their origin in the Declaration of Helsinki and will adhere to the Ethical and Regulatory Considerations as stated. I confirm that if I or any of my staff are members of the Institutional Review Board, we will abstain from voting on this protocol, its future renewals, and its future amendments.

Principal Investigator Name: _____

Principal Investigator Signature: _____

Date: _____

THIS PAGE IS INTENTIONALLY BLANK

Contents

List of Tables

List of Figures

Abstract

Although more than a half-century has passed since the therapeutic use of corticosteroids was first reported, lack of consensus continues regarding the diagnostic criteria defining adrenal insufficiency and the corresponding target population for corticosteroid replacement therapy, particularly in the setting of critical illness. Since more than 90% of circulating cortisol in plasma is protein bound, changes in the concentration of cortisol binding proteins that commonly occur with critical illness will alter measured serum total cortisol, without affecting the concentration of free cortisol, the biologically active form of the hormone. Accordingly it has been suggested that free plasma cortisol may provide a better index of adequacy of the cortisol stress response, because it corresponds more closely with intensity of illness severity. Assessment of free cortisol is currently hampered by time consuming equilibrium dialysis isolation of the free cortisol fraction. No information regarding free cortisol kinetics has been compiled for the population of critically ill children. Cortisol Quantification Investigation (CQI) is a prospective, observational cohort study that will assess baseline total and free serum cortisol concentrations in a convenience sample of critically ill children exhibiting a spectrum of illness severity. This investigation will be conducted among seven performance site pediatric intensive care units comprising the NICHD Collaborative Pediatric Critical Care Research Network. As an overall hypothesis, the study surmises that free, plasma cortisol fractionated by temperature controlled ultrafiltration is equivalent to free plasma cortisol fractionated by the gold standard equilibrium dialysis technique when a common assay method (immunochemiluminescence) is utilized.

1 Study Summary

1.1 Specific Aims

Specific Aim 1. Confirm that free plasma cortisol fractionated by temperature - controlled ultrafiltration and measured by immunochemiluminescence is highly correlated with free cortisol fractionated by equilibrium dialysis and measured by immunochemiluminescence.

Hypothesis 1. Plasma free cortisol fractionated by equilibrium dialysis (gold standard) will be very highly correlated with plasma free cortisol fractionated by temperature-controlled ultrafiltration when both fractions are assessed using an immunochemiluminescence assay.

Specific Aim 2. As a proof of principle pilot investigation, we will examine single nucleotide polymorphisms in genes within the cortisol biosynthesis regulatory network to identify those SNPs that are associated with low cortisol synthesis in response to stress.

Hypothesis 2. Baseline plasma free cortisol levels in critically ill children will be significantly associated with certain polymorphisms in genes regulating cortisol biosynthesis and degradation. These associations will be elucidated in a candidate gene study encompassing 31 genes involved in cortisol response.

1.2   Patient Eligibility

1.2.1   Inclusion Criteria

Patients will be eligible for enrollment if:

- they are admitted to a pediatric intensive care unit (PICU); AND

- they are greater than 40 weeks gestation and less than 18 years of age; AND

- they are greater than 5 kg in weight; AND

- parental consent and blood sampling can be accomplished within 24 hours of admission to the PICU; AND

- they have a vascular catheter capable of providing blood samples.

1.2.2   Exclusion Criteria

Patients will be ineligible for enrollment if ANY of the following is present or anticipated:

- systemic steroid administration within previous month; OR

- lack of commitment to aggressive intensive care therapy; OR

- subject is status post cardiopulmonary bypass, extracorporeal membrane oxygenation (ECMO), leukopheresis, plasmapheresis, or massive transfusion ($> 50\%$ of total blood volume); OR

- subject unexpected to survive PICU admission; OR

- subject previously enrolled in this study; OR

- ketoconazole administration within previous month; OR

- etomidate administration within previous month.

1.3 Anticipated Recruitment and Study Duration

This study will recruit a convenience sample of approximately 140 to 200 subjects. Subjects will be recruited across a spectrum of severity of illness as measured by the PRISM III score.[1] Each clinical site will request 5 to 10 patients in each of the following PRISM quartiles: 0 to 7, 8 to 15, 16 to 23, and greater than or equal to 24. It is anticipated that patient accrual for this study will require one year.

1.4 Inclusion of Women and Minorities

Study subjects in this project are entirely infants and children admitted to Pediatric Intensive Care Units in CPCCRN clinical centers. The gender, ethnic and racial composition of patients enrolled in all CPCCRN studies is a function of the underlying referral population at the clinical centers selected by the National Institute for Child Health and Human Development (NICHD) to participate in the network. During this study, the Data Coordinating Center (DCC) will monitor patient accrual by race, ethnicity, and gender. If necessary, additional recruitment efforts will be made at specific centers to ensure that the aggregate patient sample contains appropriate gender and minority subsets.

2 Background and Significance

2.1 Hypothalamic-Pituitary-Adrenal Axis in Sepsis

Intense interest in the role of adrenal hormones in the pathophysiology of severe sepsis has persisted since the first description of apoplexy of the adrenal glands in septic shock roughly one century ago.[2] Most critical care research investigating adequacy of the adrenal stress response has focused on severe sepsis/septic shock. Initial response to infection is mediated through the innate immune system via elaboration of multiple protein and lipid mediators, which subsequently activate a neurological - endocrinological - inflammatory stress response,[3–6] which includes stimulation of the hypothalamic - pituitary - adrenal (HPA) axis resulting in increased cortisol production.[7,8] This

complex biochemical orchestration reflects coordinated activity of the entire HPA axis and integrated neurological - inflammatory - endocrinological signaling. Stress signals associated with severe sepsis overcome the normal negative feedback regulation of the HPA axis.[5–9]

2.2   Defining Stress-Associated Adrenal Sufficiency

Defining appropriate adrenal status in the setting of critical illness stress currently requires invocation of relativity if not existentialism. Despite significant clinical research in this area, there is no consensus on what constitutes an adequate adrenal response to severe stress.[10] In the case of sepsis several inflammatory biochemical mediators likely contribute to reduced adrenal responsiveness including: defensins with corticostatin activity,[11,12] human neutrophil polypeptide-4,[13] ACTH-like peptides,[14] transforming growth factor,[15] macrophage migration inhibitory factor,[16] and tumor necrosis factor.[17]

Results of the largest, prospective interventional trial ($N = 599$) of stress dose adjunctive corticosteroid therapy for severe sepsis in adults, CORTICUS, found no differences in 28-day all-cause mortality between subjects receiving placebo or hydrocortisone, and this result was consistent in subgroup analysis examining subjects displaying an adequate adrenal stress response versus those exhibiting so called relative adrenal insufficiency (corticotropin-stimulated minus basal total serum cortisol $< 9\ \mu g/dL$).[18] On the other hand, the group receiving hydrocortisone demonstrated faster resolution of their shock, but increased hyperglycemia, nosocomial infections, sepsis and septic shock. The investigators have concluded that hydrocortisone cannot be recommended for routine therapy of severe sepsis, nor can the traditional corticotropin stimulation test (assessed with measurement of total cortisol) be recommended to guide hydrocortisone therapy.

As noted above, comparison of studies correlating adrenal status with outcome are hampered by lack of consensus regarding what actually constitutes a sufficient versus insufficient adrenal response to intense stress.[10,19] Moreover, quantification of total versus free cortisol will generate different answers particularly in patients with hypoalbuminemia.[20] Even in the presence of elevated plasma cortisol, relative local cortisol insufficiency may occur at sites of inflammation (corticosteroid resistance syndrome) secondary to: 1) depletion of corticosteroid-binding globulin; 2) activation of 11-beta-hydroxysteroid dehydrogenase; 3) depression of glucocorticoid receptors; 4) diminution of receptor affinity for cortisol; and 5) elevation of anti-glucocorticoid compounds or receptors.[21]

2.3 Laboratory Assessment of Adrenal Function

Historically adrenal function has been assessed by quantification of blood total cortisol concentration, typically at baseline and then following adrenal stimulation with exogenous corticotropin. However, an evolving consensus stresses that free rather than protein-bound (albumin and transcortin) cortisol is responsible for the biochemical actions of this hormone.[22–24] This may be particularly important in critical illness when alteration in the concentrations of cortisol binding proteins are known to occur.[20] Normally cortisol binding globulin and albumin transport over 90% of circulating cortisol. During critical illness the concentrations of these proteins may decrease by 50%, but individual variation is marked.[25] For example serum albumin is translocated into interstitial space in the setting of diffuse capillary leak, and transcortin may be cleaved by neutrophil elastase increasing directed delivery of free cortisol at foci of inflammation.[26] In a study of 66 critically ill adults who exhibited a marked increase in serum free cortisol, approximately 40% would have been judged as relative adrenal insufficient at baseline and following corticotropin stimulation based on total serum cortisol concentrations.[20] In critically ill adults with sepsis and septic shock, free cortisol corresponds more closely to illness severity than total cortisol.[25]

To measure free cortisol, it is necessary to fractionate the free cortisol from protein-bound cortisol, and then to assay the fractionated cortisol. Isolation of the free cortisol fraction has generally been accomplished by either equilibrium dialysis or ultrafiltration, performed under controlled temperatures, as cortisol binding to proteins is temperature dependent.[27] Equilibrium dialysis is considered the gold standard fractionation methodology for comparison. Quantification of cortisol has been achieved using radioimmunoassay, chemiluminescence assay, ELISA, fluorescence polarization immunoassay and mass spectroscopy. Each assay generates a different reference range. Traditionally free cortisol has been assayed following equilibrium dialysis or ultrafiltration/ligand binding methods, that are hampered by expense, technical difficulties, large volume serum requirement, long analysis times, and need for a radionuclide.[28]

More recently, free cortisol has been measured following temperature controlled ultrafiltration of serum samples[29] without ligand binding. This alternative approach avoids radioactivity, is much faster, and accommodates smaller serum volumes. A recent comparison of ultrafiltration and equilibrium dialysis fractionation methodologies concluded that both produce acceptable reproducibility with very similar results when the filtrate of dialysate is assessed by the same automated immuno-analyzer system.[30]

Free cortisol has not been assessed in critically ill children.

2.4   Genetics of Cortisol Metabolism

Variability in the cortisol response to critical illness may arise from different levels of stimulation (i.e. greater or lesser severity of illness) or it may be attributable to genetic differences between patients. A meta-analysis of 5 comparable twin studies indicates that 62% of the individual differences in basal cortisol levels are attributable to genetic effects.[31] Recently, studies of single nucleotide polymorphisms (SNPs) indicate that SNPs in genes involved in cortisol synthesis or in regulatory control of cortisol synthesis may be partially responsible for differences in basal cortisol levels.[32–34] At this time, very little has been done to examine the effects of genetic variation on cortisol synthesis during physiologic stress. To gain a more complete understanding of the contribution of genetic effects on cortisol levels measured during physiologic stress, we propose to examine a network genes involved in regulatory control and synthesis of cortisol.

The endocrine system regulating cortisol secretion consists of at least 31 genes (Figure 5 on page 35 shows a network diagram of interactions among these genes, and additional information about the candidate genes is listed in Table 2 on page 36). The protein products of these genes serve a variety of functions in the regulation of cortisol synthesis. The best known of this group are genes are those directly responsible for the synthesis of cortisol in the adrenal cortex as indicated in Figure 5 on page 35. Control over cortisol synthesis begins with the synthesis of corticotropin releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus. The synthesis of CHR is in part regulated by negative feedback from cortisol levels which is modulated by a nuclear transcription factor (NR4A2). Newly synthesized CRH binds to its receptor (CRHR1), causing cells in the anterior lobe of the pituitary to secrete pro-opiomelanocortin (POMC) which undergoes post-translational processing to become ACTH. At the adrenal cortex, ACTH binds to its receptor (MC2R) which, in conjunction with MRAP triggers an intracellular signaling cascade beginning with G proteins. These activate adenylyl cyclase which regulates the intracellular machinery responsible for the synthesis of cortisol.

2.5   Summary and Rationale for the Study

The proposed CQI will assess plasma free cortisol concentrations at baseline in a population of critically ill children with a spectrum of illness severity.

We will determine whether plasma ultrafiltration can be used in place of equilibrium dialysis by comparing the measured free cortisol following each fractionation method. If immunochemiluminescence assay following plasma ultrafiltration yields the same results as following equilibrium dialysis, then we will be able to measure free cortisol levels within a few hours. This will enable feasible measurements of free cortisol in critically ill children in subsequent CPCCRN studies of adrenal function. These measurements will also provide the first direct measurements of free cortisol in critically ill children.

As a pilot investigation in these same patients, genotyping of a tag set of SNPs in and near candidate genes responsible for cortisol regulation also will be performed. If the SNP variability in cortisol regulation is significantly associated with plasma free cortisol levels, then future CPCCRN studies of sepsis, septic shock, and adrenal function may need to be stratified for meaningful comparison of treatment arms and/or potentially effective trial treatments might depend on the patients' genotypes.

3   Preliminary Studies

Although evolving consensus suggests quantification of free cortisol in preference to total cortisol for assessing relative adrenal insufficiency or inadequate adrenal reserve,[35] current equilibrium dialysis methodology has multiple limitations as noted above. Preliminary studies focused on development of a methodology to provide rapid, sensitive, accurate plasma free cortisol concentrations have been conducted with the Clinical Laboratory at Seattle Childrens Hospital. Both total and free cortisol have been measured utilizing the Ortho Clinical Diagnostics VITROS® Immunodiagnostic System, that permits stat, random access capability utilizing enhanced chemiluminescent detection. A horseradish peroxidase-labeled cortisol conjugate competes with sample cortisol for binding to a biotinylated sheep anti-cortisol antibody. Antigen-antibody complexes are subsequently captured on streptavidin- coated wells to which a luminogenic substrate is added. Horseradish peroxidase-labeled conjugates bound to the streptavidin coated wells generate chemiluminescence that is inversely proportional to substrate cortisol.

Fractionation of plasma free cortisol has been accomplished utilizing temperature-controlled ultrafiltration, essentially according to the method of Lentjes, et al[29,30] employing Millipore YM-30 membrane filters (molecular weight cutoff 30,000). Basically serum samples are subject to temperature-

	0800		1600	
	Total	Free	Total	Free
Mean	10.48	0.97	6.63	0.56
S.D.	5.06	0.38	2.67	0.22

Table 1: Cortisol concentrations, expressed as $\mu g/dL$

controlled (37°) centrifugation to yield a protein poor free cortisol fraction that can be analyzed using the chemiluminescent immunoassay described above. This ultrafiltration fractionation methodology was compared to more traditional equilibrium dialysis fractionation. Linearity of the method (temperature-controlled ultrafiltration and chemiluminescence immunoassay) was first established by assaying serial crystalloid dilutions of a patient serum sample with known high free-cortisol concentration. Correlation of expected (calculated) results with assay results was found to be excellent with $R^2 = 0.99$ and a corresponding least squares equation: $y = 1.18x + 0.18$.

Intraassay variation ($n = 10$ assays each) was determined at two free cortisol levels, 0.70 and 7.00 $\mu g/dL$, and found to be 0.70 ±0.056, CV 7.98% and 7.01 ±0.200, CV 2.85% respectively. Interassay variation ($n = 15$ assays) was also determined at two free cortisol levels, 0.6 and 6.0 $\mu g/dL$, over a 28 day interval and found to be 0.57 ±0.13, CV 23.7% and 6.25 ±0.38, CV 6.05% respectively.

Total and free serum cortisol concentrations were determined on a convenience sample of 22 normal, unstressed, control individuals at 0800 and 1600 and are summarized in Table 1 above.

Seattle Childrens Hospital reference range for normal for total serum cortisol measured by chemiluminescence immunoassay is $4.5 - 23.0$ for a.m. samples and $\approx 50\%$ this for p.m. samples. For free serum cortisol levels, Quest Diagnostic (http://www.questdiagnostics.com) reports ranges of free cortisol of $0.40 - 1.92$ for a.m. samples and $0.20 - 0.90$ for p.m. samples, utilizing equilibrium dialysis fractionation and radioimmunoassay. Free cortisol represented $9.71 \pm 2.76\%$ of the total cortisol for this control cohort.

Free cortisol levels were determined simultaneously by (1) ultrafiltration and chemiluminescence immunoassay; and (2) Quest Diagnostics equilibrium dialysis and radioimmunoassay in another group of 20 control samples. Correlation between the two assays was good with $R^2 = 0.81$.

Subsequently serum cortisol was assessed in 20 critically ill children with various diagnoses and a spectrum of illness severity (PRISM III 13.3 ± 9.8, range 2-38; PELOD 11.4 ± 11.6, range 0-40; and vasoactive-inotropic

score 17.0 ± 19.3, range 0-70.) Baseline total serum cortisol for this cohort averaged 20.4 ± 13.4, range 4.2-47.1 $\mu g/dL$. Ten subjects demonstrated baseline total cortisol concentrations < 18 $\mu g/dL$, mean 9.6 ± 4.5, range 4.2-16.3. Following corticotropin adrenal stimulation (45 minutes after $145\mu g/kg$, maximum $250\mu g$ intravenously) total serum cortisol increased to 35.8 ± 19.1, range 5.3-95.8 $\mu g/dL$ ($p = 0.0002$). Mean Δ total cortisol was 16.4 ± 13.8, range -6.3 to 53.2. Four subjects failed to increase total serum cortisol by ≥ 9 $\mu g/dL$ following corticotropin, but none of these were among the nine with baseline cortisol $< 18\mu g/dL$.

Baseline-free cortisol determined by ultrafiltration/chemiluminescent immunoassay in this group averaged 3.1 ± 3.5 (range 0.2-11.3) accounting for $11.6 \pm 7.4\%$ (range 3.9-28.2%) of total cortisol. Note that these free cortisol levels reflect a $\approx 300 - 500\%$ increase over free cortisol levels observed in non-stressed control subjects discussed above. Following corticotropin adrenal stimulation, free serum cortisol determined by ultrafiltration and chemiluminescence immunoassay increased to 8.0 ± 6.9 (range 2.3-29.7 $\mu g/dL$) (p=0.00084) now representing $26.2 \pm 11.2\%$ (range 7.9-42.8%) of total cortisol ($p = 3 \times 10^{-6}$).

Mean change in the percent of free cortisol following corticotropin was $16.7 \pm 7.7\%$ (range 3.0-30.8%). Only one subject out of 20 failed to increase serum free cortisol to > 3.1 $\mu g/dL$ following corticotropin, a value previously recommended as a possible threshold for defining normal corticotropin-stimulated free cortisol in critically ill adult patients.[20] Basal free cortisol determined by an equilibrium dialysis/radioimmunoassay method (Quest Diagnostics) averaged 3.8 ± 3.0 (range 0.7-10.3). Comparison of the ultrafiltration and chemiluminescence method with the gold standard equilibrium dialysis and radioimmunoassay method is displayed In Figure 1 on the next page. Results of the two methods are highly correlated, but utilization of the ultrafiltration and chemiluminescence immunoassay method can provide the same information with a markedly faster turn around time.

In the proposed CQI study, direct comparison of ultrafiltration versus equilibrium dialysis fractionation of free cortisol will occur utilizing the same assay method (chemiluminescence immunoassay) to subsequently measure cortisol in the resulting ultrafiltrate or dialysate samples.

Next cortisol concentrations were correlated with degree of systemic stress utilizing the various markers of illness severity noted above. Only mild-modest correlation was appreciated between PRISM III scores and either serum baseline total (0.41) or free cortisol (0.46) respectively. Even less correlation was appreciated when serum baseline total and free cortisols were paired with simultaneously determined organ dysfunctions, PELOD
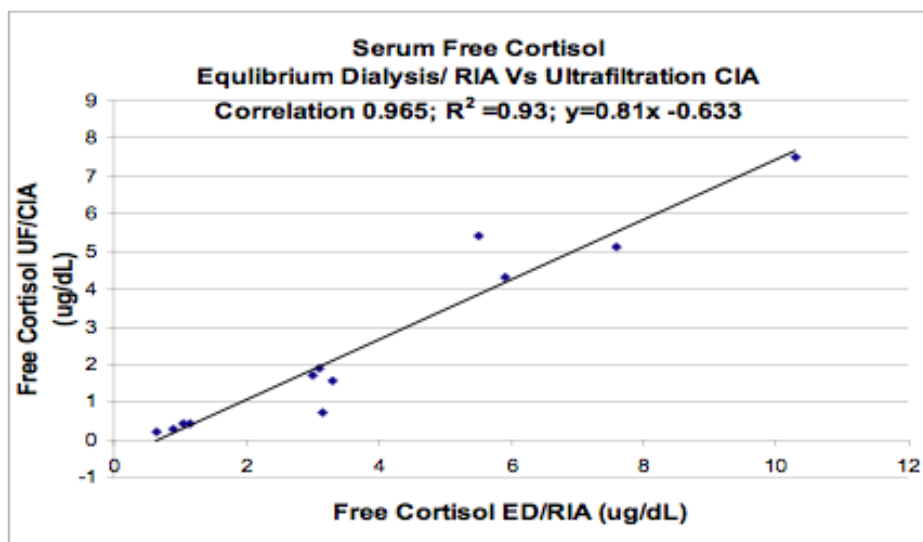
Figure 1: Serum Free Cortisol

score, or vasoactive-inotropic infusion scores (data not shown).

This group of critically ill children was approximately evenly divided between nine with systemic inflammatory response syndrome (SIRS) and eleven without SIRS. Those with SIRS tended to exhibit higher illness severity with PRISM III scores of 18.2 ± 10.9 versus 9.5 ± 7.3, $p = 0.052$; PELOD scores of 17.6 ± 13.6 versus 8.0 ± 7.3, p = 0.097; and vasoactive-inotropic infusion scores of 33.1 ± 17.2 versus 6.1 ± 8.0, $p = 0.002$. Mean basal free cortisol levels were similar ($p = 0.734$) between children with SIRS, 2.8 ± 3.4, range 0.2-10.5 versus those without SIRS, 3.4 ± 3.7, range 0.4-11.3. Similarly basal free cortisol as a percent of the total cortisol appeared to be nearly identical ($p = 0.99$) comparing children with SIRS, $11.61 \pm 7.96\%$ versus those without SIRS, $11.65 \pm 7.65\%$. However, among children with SIRS, good correlation was noted between basal total cortisol and PRISM III scores (0.848, $p = 0.008$) and between basal free cortisol and PRISM III scores (0.862, $p = 0.006$). These relationships are graphically depicted in Figures 2 and 3 on the facing page. Similarly among children with SIRS, modest correlation was noted between basal free cortisol and PELOD scores as indicated in Figure 4 on page 18.

In comparison, among children without SIRS, PRISM III and basal total cortisol correlation was 0.178 and PRISM III and basal free cortisol correlation was 0.199. No significant correlation was noted between PELOD scores
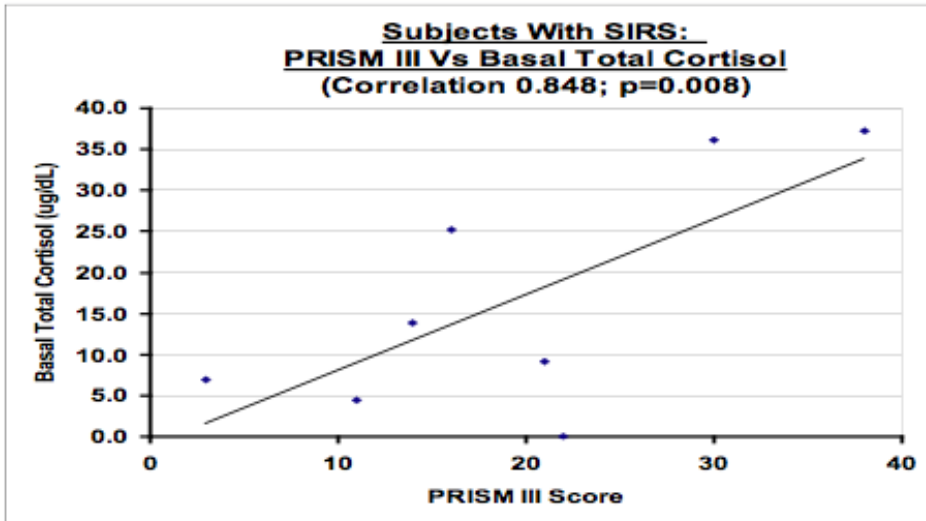
Figure 2: PRISM III vs Basal Total Cortisol
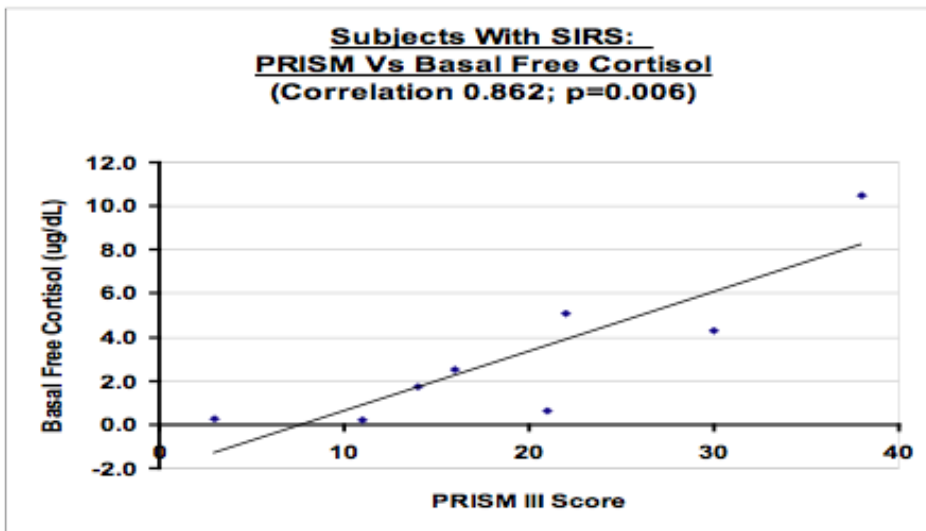
Figure 3: Prism vs Basal Free Cortisol
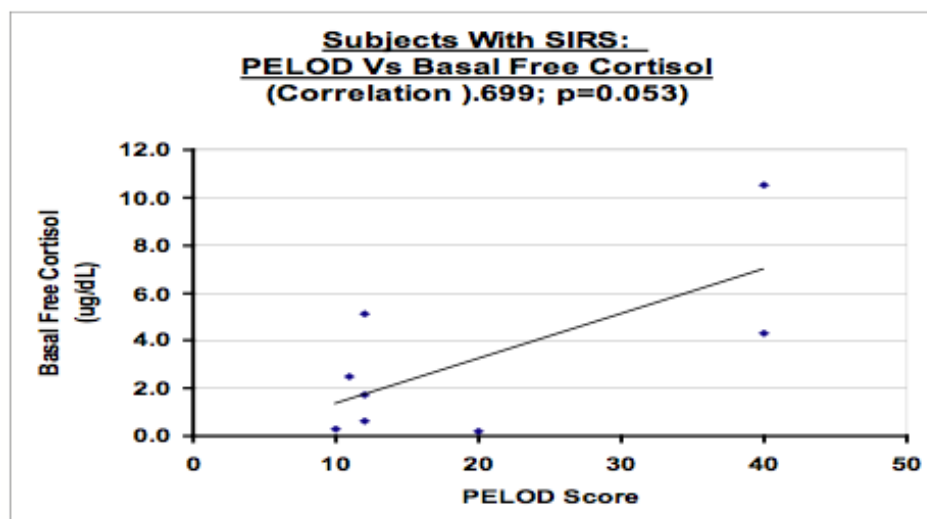
Figure 4: PELOD vs Basal Free Cortisol

and basal free cortisol for children without SIRS (0.568, $p = 0.087$) nor between vasoactive-inotropic infusion score and basal free cortisol for either children with SIRS (-0.206, $p = 0.595$) or children without SIRS (-0.367, $p = 0.297$). Although a significant relationship appears to exist between indices of illness severity among children with critical illness complicated with SIRS, a much larger cohort is required to validate this assertion.

4   Study Methods

CQI is a prospective, observational cohort study examining two fractionation methods for plasma free cortisol in relation to illness severity. Plasma cortisol will be measured as total cortisol as well as free cortisol fractionated by temperature controlled ultrafiltration[29, 30] or equilibrium dialysis[30] and quantified by chemiluminescent immunoassay. A convenience sample of children admitted to seven CPCCRN PICUs demonstrating spectrum of illness severity as assessed by PRISM III will represent the target enrollment population for this study.

Following informed parental permission and assent when applicable, each subject will be assigned a unique study number. Subsequently each subject will be evaluated for the presence of systemic inflammatory response syndrome (SIRS)[36] and basic demographic data will be collected for each sub-

ject. Clinical illness severity will be assessed utilizing the Pediatric Risk of Mortality (PRISM III) Score.[1] Primary and secondary diagnoses prompting PICU admission will be recorded. All clinical data will be recorded into TrialDB, which is the electronic database used by the Data Coordinating Center for CPCCRN.

Parental permission and blood sampling must be accomplished within the first 24 hours of admission to the PICU. The blood sample (4 mL) will be drawn from an existing vascular catheter, and the blood will be processed in preparation for batch analyses. The details of handling the blood will be provided in the study Manual of Operations.

Specific Aim 1. Confirm that free plasma cortisol fractionated by temperature - controlled ultrafiltration and measured by immunochemiluminescence is highly correlated with free cortisol fractionated by equilibrium dialysis and measured by immunochemiluminescence.

Hypothesis 1. Plasma free cortisol fractionated by equilibrium dialysis (gold standard) will be very highly correlated with plasma free cortisol fractionated by temperature-controlled ultrafiltration when both fractions are assessed using an immunochemiluminescence assay.

Total plasma cortisol will be determined directly on plasma by immunochemiluminescence assay. Free plasma cortisol fractions will be obtained by equilibrium dialysis at $37\,^{\circ}\text{C}$ utilizing the Pierce Rapid Equilibrium Dialysis Device (RED) or temperature-controlled ultrafiltration at $37\,^{\circ}\text{C}$ employing Millipore YM-30 membrane filters.[29, 30] These fractions will be assayed with the same immunochemiluminescence assay used for total cortisol measurement.

We will use Tukey mean-difference plots (Bland-Altman plots)[37]) to evaluate the agreement between the two fractional schemes. A Pearson correlation coefficient will also be estimated to evaluate the strength of the relationship

As an exploratory analysis, we will determine the relationships between plasma total and free cortisol and illness severity as quantified by PRISM III.[1] This same relationship will be further explored in subgroup analysis for subjects demonstrating evidence of SIRS.[36] In each case, we will calculate Pearson correlation between plasma cortisol concentrations and PRISM III scores.

Specific Aim 2. As a proof of principle pilot investigation, we will examine single nucleotide polymorphisms in genes within the cortisol biosyn-

thesis regulatory network to identify those SNPs that are associated with low cortisol synthesis in response to stress.

Hypothesis 2. Baseline plasma free cortisol levels in critically ill children will be significantly associated with certain polymorphisms in genes regulating cortisol biosynthesis and degradation. These associations will be elucidated in a candidate gene study encompassing 31 genes involved in cortisol response.

As indicated above, we will focus on SNPs in the 31 genes involved in the cortisol response (synthesis, regulation and feedback). Our SNP coverage will include introns and exons within each gene and SNPs located 2000 base pairs upstream (5') and 2000 base pairs downstream (3') from each gene. To select SNPs, we employed the Genome Variation Server database which provides a tool for rapid access to human genotype data found in dbSNP.

With the exception of the gene PRKACA, the SNPs were selected from the HapMap database. The gene PRKACA has recently been resequenced as a part of the NHLBI Program for Genomic Applications (PGA) project. Because this database contains a higher density of SNPs the than the HapMap database, information from the PGA database was used for this gene. To maintain good statistical power, we limited SNP selection to those with a minor allele frequency of $\geq 5\%$,[38] which yielded a total of 1165 SNPs for the 31 genes and their flanking regions. To make genotyping affordable while maintaining good coverage of the candidate regions and reducing the effects of multiple testing in the statistical analysis, we employed a tag SNP approach to select a subset of these 1165 SNPs for typing. Tag SNPs were selected such that no SNP in the tag set had an R^2 value of < 0.80 with a least one other SNP, yielding a total of 344 tag SNPs. The algorithm used for selecting tag SNPs is guaranteed to fulfill this correlation condition only if tag SNPs are selected from a single population.[39] Because the non-Hispanic white population constitutes the largest ethnic group within the population served by the CPCCRN ICUs (42%) and is less diverse in terms of polymorphisms than that for the next largest group (African Americans, 25%), we selected SNPs from the non-Hispanic white population. Because the African American population has a higher density of SNPs, this tag set is expected to provide even better coverage for this population. The pattern of polymorphisms in the Hispanic population (22%) is related closely enough to that of non-Hispanic white population so that adding another tag SNP set for this population would probably provide less power to detect effects with the small sample size of 140 patients; it would increase the number

of multiple tests performed and decrease the overall statistical power while increasing costs. We will control for potential population stratification in the regression analysis described below in the statistical analysis section. All other ethnic groups comprise 5% of the CPCCRN ICUs population and cannot be studied separately with the sample size of 140 patients.

Nucleic Acid Isolation and Genotyping Analysis. Tubes containing the plasma-mononuclear leukocyte fraction will be thawed, and then centrifuged at 1000 x g RCF for five minutes to precipitate the mononuclear cells. The cell pellet will be resuspended in 0.5 mL of PBS by vortexing or pipetting. DNA and RNA will be isolated from these samples using a commercially available silica-based system (AllPrep DNA/RNA system Qiagen). The RNA will be frozen and stored for potential future investigations (see Section 7.3 on page 30). DNA quality will be assessed spectrophotometrically by measuring the OD 260/280 ratio. A ratio of greater than 0.8 will be deemed satisfactory.

SNP genotyping. SNP genotyping will be performed by Dr. Deborah Nickerson's laboratory in the Department of Genome Sciences at the University of Washington. Genotyping will be performed using the Illumina Custom Golden Gate 384 Plex assay.[40] This system allows for multiplex assays (384-plex) with high sample throughput (96 samples). Full processing of samples using the Golden Gate protocol requires 2 days to complete. On day one, normalized sample DNA is activated with biotin and captured with streptavidin conjugated paramagnetic beads. Allele-specific oligos and linker oligos are hybridized to the biotinylated DNA. Allele-specific extension and ligation is performed on the hybridized oligos. Subsequently, PCR incorporating Cy3 and Cy5 dyes is carried out on the extended and ligated products. On day two, the fluorescent labeled PCR products are denatured and single-stranded, labeled product is hybridized to microbeads in a 96-well plate format. Each silica bead is specific for one SNP and is identifiable by an addressing algorithm. The SNP allele is determined by the color of the flurophore bound to the bead. Each well containing microbeads can assay 384 sites per well. Hybridized microbeads are scanned using the Illumia BeadXpress scanner, a dual-color detection system. Genotypes are called using the Illumina BeadStudio software package, which normalizes and clusters the raw scan data. Numerous assay controls are built-in to each oligo pool for allele-specific ligation, PCR uniformity, gender, extension and gap ligation, hybridization, and oligo contamination.

Statistical Design Considerations and Statistical Analysis. The lead statistician for Aim 2 is Dr. Mary Emond from the University of Washington Department of Biostatistics. The design and statistical analysis of this study will draw heavily on the careful planning and extensive research done by investigators of the Wellcome Trust Case Control Consortium.[41] Both theoretical and applied statistical methods have been developed and refined in the context of this consortium to deal with the issues ranging from the theory of multiple testing[41] to application of methods for controlling for population structure, differential bias and technical miscalls of heterozygotes.[42] Numerous software packages have been developed and made public for implementation of the methods used by the Consortium.[41] The method of Bayes factors was used by the Consortium to determine the posterior probability (a kind of Bayesian significance level) that a SNP is associated with disease. This method was developed earlier by Desai and Emond in the context of determining somatic single nucleotide changes significantly associated with the development of cancer.[43] Dr. Emond has also completed research on increasing statistical power in genome-wide studies.[44]

A superficial difference between the major SNP/disease-association studies published by the Wellcome Trust Consortium and the currently proposed study is that the phenotype in this study is a quantitative trait (cortisol level), whereas the phenotype for most Wellcome Trust studies published to date has been disease status, a naturally discrete trait (present versus absent). However, from the statisticians point of view, this difference reduces to a difference in the models used: for the quantitative phenotype, regression models and tests of association between the continuous outcome and genotype are used, while logistic regression (or equivalent chi-square tests) are used for the discrete outcome. Statistical power is lost by discretizing a continuous phenotypic measure into arbitrarily defined cases and controls,[45] even though examples of this practice appear in the prestigious biomedical journals. In the study proposed here, all primary analyses will be performed with cortisol as a continuous dependent regression variable with genotype as the independent variable of interest.

Design considerations. Genotyping will be done after all samples are collected, in the shortest time window possible in order to avoid technical error drift. To avoid irreconcilable confounding by center and ethnicity (population structure), samples will be randomized to experimental day by center and ethnicity of the contributing patient.

Analyses. The 344 chosen SNP genotype results will be tested for Hardy-Weinberg equilibrium (HWE) within ethnic groups. Unlike some statistical tests for allelic association with disease, the regression method to be used here does not depend on the assumption that the sub-populations are in HWE. However, we expect these loci to be in HWE, so this will be a test for data quality. Any loci that seriously deviate from HWE will need to be examined for data errors and/or discarded. The genotypes will then be assessed for association with cortisol level using a linear regression model with genotype included as a linear or log-linear variable coded as 1, 2, 3 for the rarer homozygote, heterozygote and more common homozygote, respectively. If chip-to-chip variation results in the need for normalization across chips, the published software for normalization of the Affymetrix chip will be modified for use with the Illumina chip. The resulting test statistics will be examined for any overdispersion that indicates systematic biases using quantile-quantile plots as described in Clayton et al[42] with any necessary adjustments done by the method of modified genomic control described therein and/or by modification of the CHIAMO algorithm to the Illumina chip. However, it is likely that Illumina will have adopted this algorithm or a more effective algorithm by the time the genotyping in this study is undertaken. A categorical variable for ethnicity will be included as a potential control for confounding by population structure, but this variable will be dropped if no shift in the association is seen (a change of 1 standard deviation or more in the coefficient for association will mean the ethnicity variable is retained.[46] Principal components of the ethnic groups will be investigated to provide a more parsimonious control variable for ethnicity and geographic origin of the patient (e.g. two levels rather than 5 to 7) as done in the Wellcome Trust major report.[41]

Categorical variables for type of illness (e.g. SIRS present vs. absent) and/or illness severity (e.g. PRISM scores) also will be examined as potential precision variables or confounding variables, since different types and severity of illnesses might provoke different cortisol responses (see above). Other potential adjustment variables include age, sex, and serum albumin level.

A SNP will be declared as significantly associated with cortisol response if the posterior odds of association with genotype are 10:1 or greater given the observed data. Desai and Emond have discussed the use of posterior odds for association of polymorphisms with disease.[43] This criterion is directly interpretable as a probability of association (unlike p-values that are probabilities under null hypotheses that don't exist when true association is present) and was again used by the Wellcome Trust Consortium[41] and found

to be in good agreement with other criteria for significance that account for multiple testing.

Statistical Power.   Power is defined as the probability of declaring a significant association given that a true biological association exists. The statistical power depends on the strength of the biological association, the minor allele frequency (MAF), the sample size and the efficiency of the statistical methods used. As noted above, using a continuous outcome is the most efficient statistical methodology for the CQI study. The sample size is already fixed at 140 subjects. Hence, the power for finding at least one significant association will be determined by the number and magnitude of the biological associations present as well as the MAFs. An especially nice aspect of this chosen tag SNP set of 344 is that MAFs among them average about 0.30, which is quite high (providing good power) compared to the MAFs thought to be involved in many common complex diseases such as coronary artery disease. We will conjecture that about 5 of the 344 tag SNPs will be associated with cortisol levels, either directly through causation or indirectly through linkage with nearby causative polymorphisms. This is a reasonable assumption, since the cortisol pathways have a long history of study and are well-understood: some of the 31 selected candidate genes are highly likely to be significantly involved in cortisol regulation in severely ill children. This is contrast to the disease states studied by the Wellcome Trust Consortium, where multiple unknown pathways from anywhere in the genome may be involved but unknown to science at this time. For example, pathological mechanisms related to Crohns disease are poorly understood. Nevertheless, previously discovered associations such as CARD15/NOD2 and the region on 5q13 tagged by rs6596075 were reproduced in the Wellcome Trust Study, in addition to finding new associations that are scientifically highly plausible. This represents a re-assuring sign of the validity and care in the Wellcome Trust Consortium study design and analysis. With this conjecture, a p-value of 0.0012 would be needed to declare significance after testing 344 SNPs. Based on the preliminary cortisol data from the 20 critically ill children (Section 3 on page 14), one can calculate the necessary slope to provide this p-value with 80% power. After log-transformation of the right-skewed free cortisol levels, the mean and standard deviation are 0.28 and 0.54 $log(\mu g/dL)$, respectively. For an MAF of 0.30, this translates to a (re-transformed) difference of $2\mu g/dL$ for each increment in genetic score required to achieve 80% power. (The standard genetic score is defined as 1=aa, 2=aA, 3=AA; calculations were done via

computer simulation.) An average difference of at least $2\mu g/dL$ for between groups with different genotypes at a locus is well within biological plausibility. Hence, this study has good power to detect a plausible biological effect. Smaller effects will be associated with lower power and vice versa. Power can also be increased by lowering the criteria for signficance to be less than 10 to 1 odds in favor of association. This is appropriate for a pilot study, since it is more desirable to include a few false positives in a larger follow-up study or replication study than it is to miss important true positive results. Since this is a pilot study where preliminary genotype data are currently lacking, further conjecture regarding the power is probably of little additional value here. The basic conclusion is that this study can detect effects of a reasonable magnitude, but it remains to be seen how large the effects are in reality. Analyses will be done using specially developed SNP analysis functions in the statistical package R (www.r-project.org) and Illumina software when appropriate.

5   Data Management

Clinical data will be collected at the time of enrollment. Laboratory data will be derived from analysis of the single blood sample obtained at the time of enrollment, and from the patient's medical record.

Data will be entered into TrialDB, an electronic data collection system used by the CPCCRN DCC. The paper worksheets should be retained at the clinical center in a locked file cabinet within a locked office until the study is complete and all CPCCRN publications have been accomplished. These paper worksheets will be subject to site monitoring reviews (see Section 6 on page 28).

The following data elements will be obtained and recorded when a patient is enrolled into the study, and when the laboratory results are available:

1. TrialDB Study Number

2. Clinical Center ID

3. Dates of Admission, Enrollment, and Blood Sampling

4. Date of Birth

5. Gender

6. Race

American Indian or Alaska Native A person having origins in any of the original peoples of North and South America, including Central America, and who maintains tribal affiliation or community attachment.

Asian A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent, including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam.

Black or African American A person having origins in any of the black racial groups of Africa.

Native Hawaiian or Other Pacific Islander A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.

White A person having origins in any of the original peoples of Europe, the Middle East, or North Africa.

Other (provide text) Should provide text description.

Stated as Unknown Explicitly stated as unknown.

7. Ethnicity

   - Hispanic or Latino
   - Not Hispanic or Latino
   - Stated as Unknown

8. CBC and differential (if clinically obtained)

9. Serum albumin (if clinically obtained)

10. Primary and Secondary PICU Admission Diagnoses

    - Asthma (reactive airway disease)
    - Cancer
    - Cardiac arrest w/in 24 hours (closed chest massage)
    - Chromosomal abnormality (not hereditary condition)
    - Diabetes
    - Drug overdose (e.g. ingestion, toxicity)
    - Gastroesophageal reflux
    - Cardiovascular disease - acquired

- Cardiovascular disease - congenital
- HIV infection
- Hypoxic-ischemic encephalopathy (acute, not static)
- Medical device malfunction
- Meningitis
- Pneumonia / bronchiolitis
- Seizures (includes complications of seizure therapy)
- Sepsis
- Shock
- Suicide attempt (includes intentional drug overdose)
- Transplant
- Trauma
- Other Diagnosis

11. Chronic Diagnoses

- Bronchopulmonary dysplasia (BPD)
- Cancer
- Cerebral palsy
- Chromosomal abnormality (not hereditary condition)
- Congenital heart disease
- Diabetes
- HIV infection
- Hydrocephalus
- Intraventricular hemorrhage (from perinatal period)
- Mental retardation
- Meningomyelocele / spina bifida
- Short gut syndrome
- Static encephalopathy
- Transplant
- Other Diagnosis

12. Total maximum breathing rate in first 24 hours

13. Maximum and minimum central temperature in first 24 hours

14. Maximum heart rate in first 24 hours

15. PRISM III on admission

When the subject is enrolled in the TrialDB system, a study number will be automatically provided to the clinical site. Blood samples will be labeled with the date of sample collection and the TrialDB study number. No clinical data will accompany the blood samples.

The DCC will recode the dates of enrollment and birth to calculate the subject's age, in days, and the resulting analytical database fulfills the definition of a de-identified database according to HIPAA definitions. This activity (recoding the dates to calculate age) is done under Business Associate Agreements with each clinical site (see Section 7.6 on page 31).

6   Training and Site Monitoring

CPCCRN investigators and research coordinators will receive training in conjunction with an already scheduled Steering Committee meeting. CPCCRN investigators and study coordinators will be trained to use TrialDB for entry of clinical data for this study. The TrialDB system is already in use in CPCCRN studies, and training will focus on the specific data screens developed for this study.

Site monitoring visits may be performed by staff from the DCC or the funding agency, to ensure that all regulatory requirements are being met and to monitor the quality of the data collected. During site monitoring visits, patient forms and original source documents would be inspected. The primary criterion for data element verification is identification in the source document, which is the medical record. Thus, the medical record must be available for review by the site monitor if monitoring is implemented. In addition, the site monitor would inspect the laboratory facilities that are used for processing, storing, and shipping the specimens in this study.

Remote site monitoring may also be performed by DCC staff to verify selected data elements. This will require the clinical site staff to locate the relevant source document, make a copy, de-identify the material with a black marker, and send the copy via FAX server to the DCC, where the information will be compared with data stored in the TrialDB system. Remote monitoring documentation will not be distributed outside the DCC, and will not be provided to the study investigators. The documents will be retained in accordance with Federal requirements (see Section 8 on page 32).

7 Human Subjects

This study requires approval of the Institutional Review Board (IRB) at every participating clinical site and the DCC at the University of Utah. The DCC will track IRB approval status at every clinical site, and will not permit enrollment of subjects without documentation of initial and on-going approval from each site's IRB.

7.1 Obtaining Permission for Participation

The parents, guardians or responsible family members of subjects who are eligible for this study will be provided with information about the study, including the potential risks and benefits, and written permission will be obtained for participation of the subject in the study. Assent will be obtained from subjects who are capable of providing assent, in accordance with the judgment of the Institutional Review Board (IRB) at each clinical center.

7.2 Potential Risks and Benefits

There are no major risks associated with participating in this study. Blood sampling will be carried out from an indwelling catheter, and no separate venipuncture will be performed to enable participation in this study. The total blood volume required for this study is 4.0 mL. The other potential risk is disclosure of confidential clinical information, or perceived association of the gene expression data with an identifiable individual.

There are no potential benefits for subjects who participate in this study. The results of cortisol and SNP analyses will not be available during the management of the subject, and will not be available to the clinical site. These tests will be performed in Seattle in batch mode. If patients require clinical assessment of adrenal function, such testing will be conducted in accordance with local hospital practice.

Free cortisol has not been measured in critically ill children, and the use of steroids to treat relative adrenal insufficiency is common. The results of this study will enable a more rational approach to treatment of such children with steroids, and will enable the design of rigorous randomized controlled trials for treating children with sepsis and septic shock.

The SNP analyses may help us understand why certain individuals display relatively lower plasma cortisol levels in response to stress. This information may lead to improved management of critically ill children in the

future.

7.3   Biologic Sample Retention

If biologic material is not completely consumed by the analyses of this study, remaining samples will be retained in Seattle. Seattle will not have any identifying information, and the use of residual samples will be restricted in the following manner:

1. The samples will be completely de-identified; a new study number will be assigned to each sample and the study data elements in TrialDB will be attached to this new study number. The TrialDB number will then be removed from the sample. There will be no link between the original TrialDB numbers and the new study numbers assigned to the samples. It will not be possible to link the samples back to the child, hospital, or medical records.

2. Studies must be related to cortisol response, pediatric sepsis, immune response, and other topics that are related to this original study.

3. Studies will be approved by the Steering Committee of the CPCCRN and the NICHD.

7.4   Protection Against Risks

The risk of disclosure of confidential clinical data is minimized by keeping identifiable information at the clinical site in locked offices, and by minimizing identifier information stored in the TrialDB system at the DCC. Patient names and medical record numbers will not be sent to the DCC at the University of Utah, and will not be entered into the TrialDB system. The only linkage between the final analytical clinical data, cortisol measurements, and SNP analyses will be the TrialDB study number. The analytical clinical database will contain no patient identifiers and will fulfill the definition of a de-identified dataset as defined by the Health Insurance Portability and Accountability Act (HIPAA). This analytical database will be the only one available for the analysis of the current and future derivative studies.

Following completion of this study, the de-identified clinical data will be linked to each remaining biologic specimen, and the TrialDB number will be removed from the specimens. This will completely de-identify remaining biologic specimens in accordance with Section 7.3.

7.5   Data Security

The DCC at the University of Utah has a dedicated, locked server room within its offices, and the building has 24 hour on-site security guards. The data coordinating center has a state-of-the-art computer infrastructure and coordinates its network infrastructure and security with the Health Sciences Campus (HSC) information systems at the University of Utah. This provides the data coordinating center with effective firewall hardware, automatic network intrusion detection, and the expertise of dedicated security experts working at the University. Network equipment includes three high-speed switches and two hubs. User authentication is centralized with two Windows 2003 domain servers. Communication over public networks is encrypted with virtual point-to-point sessions using secure socket layer (SSL) or virtual private network (VPN) technologies, both of which provide at least 128 bit encryption. TrialDB is the clinical trials software used at the data coordinating center in Utah, and eRoom^{TM} is used for communications about the study. TrialDB, eRoom^{TM} and other web applications use the SSL protocol to transmit data securely over the Internet.

Direct access to DCC computers is only available while physically located inside the data coordinating center offices, or via a VPN client. All network traffic is monitored for intrusion attempts, security scans are regularly run against our servers, and our IT staff are notified of intrusion alerts. Security is maintained with Windows 2003 user/group domain-level security. Users are required to change their passwords every 90 days, and workstations time out after 10 minutes of inactivity. All files are protected at group and user levels; database security is handled in a similar manner with group level access to databases, tables, and views in Microsoft SQL Server.

The investigators and staff of the data coordinating center are fully committed to the security and confidentiality of all data collected for CPCCRN studies. All personnel at the data coordinating center at the University of Utah have signed confidentiality agreements concerning all data encountered in the center. Violation of these agreements may result in termination from employment at the University of Utah. In addition, all personnel involved with data coordinating center data systems have received Human Subjects Protection and HIPAA education.

7.6   Health Insurance Portability and Accountability Act

Registration of research subjects in the TrialDB system used by the DCC at the University of Utah requires a date of birth, race, ethnicity, and gender.

These demographic data are held in database tables that are separate from coded research data (including clinical data). The demographic data are required for Federal reporting purposes to delineate subject accrual by race, ethnicity, and gender.

Additional potential identifier information includes the date of admission and birthdate. Staff at the DCC will use these dates to calculate patient age. The analytical data sets (used for study analyses and archived at the end of the study) will be de-identified, and will exclude these specific dates.

The data coordinating center produces the de-identified research data sets that will be used for all analyses in this project. Since the raw data includes potential identifiers, such as dates of birth and admission, all sites have been offered a Business Associate Agreement (BAA) with the University of Utah. The BAA explains that the data coordinating center is producing the de-identified data using the data submitted by the site, and the University of Utah assumes responsibility to preserve the confidentiality of the original data. Copies of executed Business Associate Agreements are maintained at the data coordinating center in Utah.

Patient identification at the clinical site is present in subject study files, in order to enable auditing of data quality. These data will not be sent to the data coordinating center, but will be retained in locked filing cabinets in locked offices in the clinical center itself. These records should be retained until the study data have been completely cleaned, data lock has occurred, and all primary and secondary publications have been completed. In accordance with Section 8, these records will be retained for at least 3 years after completion of the research. At that time, all records with identifying information will be destroyed.

8   Record Retention

For federally funded studies subject to the Common Rule, records relating to the research conducted shall be retained for at least 3 years after completion of the research. Completion of the research for this protocol should be anticipated to include planned primary and secondary analyses, as well as subsequent derivative analyses. Completion of the research also includes additional enrollment of subjects and processing of additional samples, if funding becomes available for these activities. Finally, completion of the research also entails completion of all publications relating to the research. All records shall be accessible for inspection and copying by authorized representatives of the regulatory authorities at reasonable times and in a

reasonable manner [45 CFR §46.115(b)].

Appendices

A   Gene Network

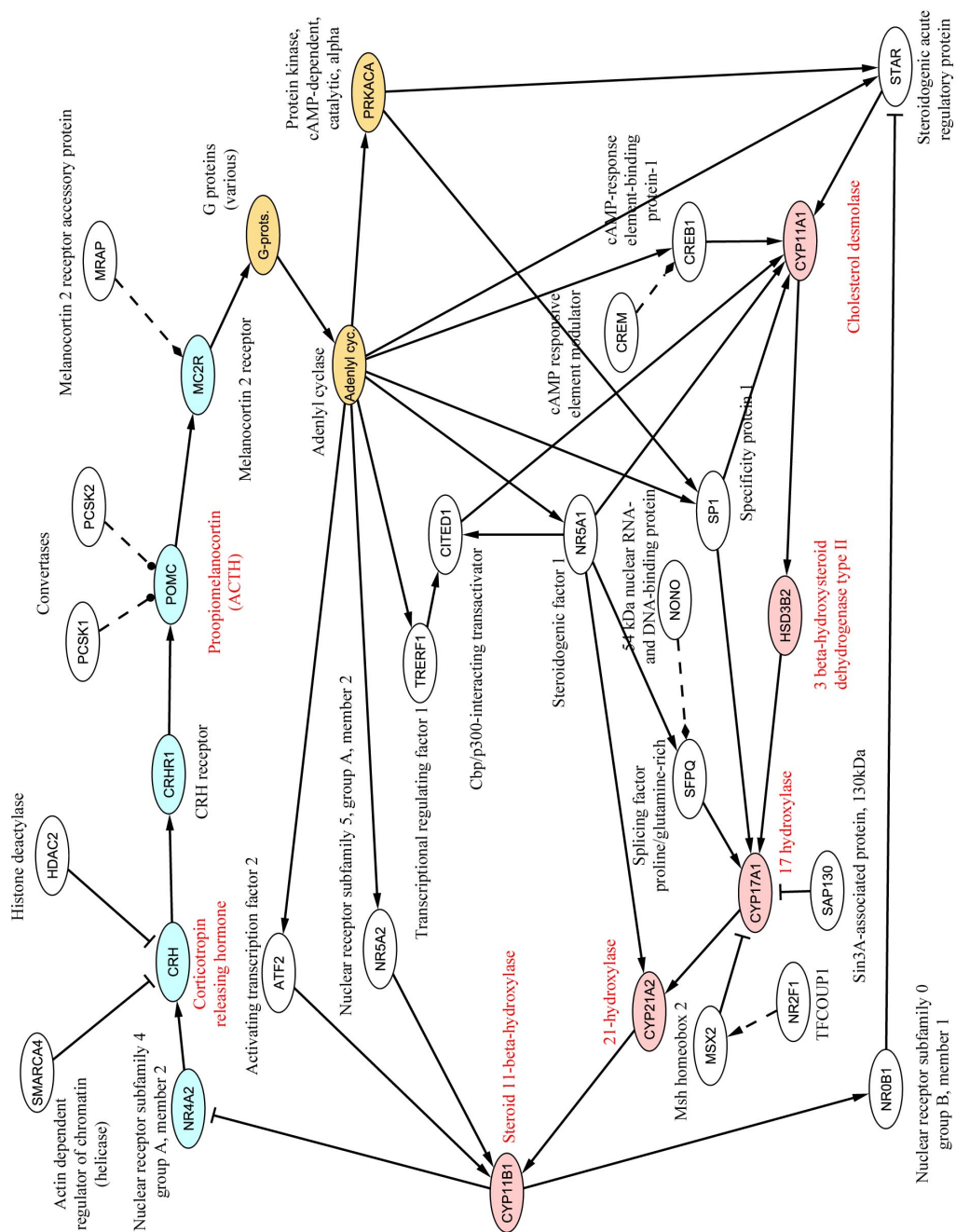See Figure 5 on the next page. This page is otherwise intentionally blank.

Figure 5: Gene Network of Cortisol Candidates

B   Candidate Gene Listing

Table 2: Candidate Genes

Gene	Name	Description
ATF2	activating transcription factor 2	This gene encodes a transcription factor that is a member of the leucine zipper family of DNA binding proteins. This protein binds to the cAMP-responsive element (CRE), an octameric palindrome. The protein forms a homodimer or heterodimer with c-Jun and stimulates CRE-dependent transcription. The protein is also a histone acetyltransferase (HAT) that specifically acetylates histones H2B and H4 in vitro; thus it may represent a class of sequence-specific factors that activate transcription by direct effects on chromatin components. Additional transcript variants have been identified but their biological validity has not been determined.
NR5A2	nuclear receptor subfamily 5, group A, member 2	Cholesterol 7-alpha-hydroxylase is the first and rate-limiting enzyme in a pathway through which cholesterol is metabolized to bile acids. The gene encoding cholesterol 7-alpha-hydroxylase, CYP7A (118455), is expressed exclusively in the liver. Overexpression of CYP7A in hamsters results in reduction of serum cholesterol levels, suggesting that the enzyme plays a central role in cholesterol homeostasis. Nitta et al. (1999) reported the identification of a liver-specific transcription factor that binds to the promoter of the human CYP7A gene. They designated this factor CPF for 'CYP7A promoter-binding factor' and identified it as a human homolog of the Drosophila orphan nuclear receptor fushi tarazu F1 (Ftz-F1). Nitta et al. (1999) isolated a CPF cDNA encoding a 495-amino acid protein from a human liver cDNA library. They found evidence for 2 CPF variants derived from alternative splicing. Northern blot analysis detected enriched expression in pancreas and liver, with a low level of expression in heart and lung.
TRERF1	transcriptional regulating factor 1	This gene encodes a zinc-finger transcriptional regulating protein which interacts with CBP/p300 to regulate the human gene CYP11A1.
CITED1	Cbp/p300-interacting transactivator	No description.

Table 2: *continued*

Gene	Name	Description
CYP11A1	cytochrome P450, family 11, subfamily A, polypeptide 1	This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the mitochondrial inner membrane and catalyzes the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of the steroid hormones. Two transcript variants encoding different isoforms have been found for this gene. The cellular location of the smaller isoform is unclear since it lacks the mitochondrial-targeting transit peptide.
HSD3B2	3 beta-hydroxysteroid dehydrogenase type II	3-beta-HSD is a bifunctional enzyme, that catalyzes the oxidative conversion of Delta(5)-ene-3-beta-hydroxy steroid, and the oxidative conversion of ketosteroids. The 3-beta-HSD enzymatic system plays a crucial role in the biosynthesis of all classes of hormonal steroids
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum. It has both 17alpha-hydroxylase and 17,20-lyase activities and is a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens. Mutations in this gene are associated with isolated steroid-17 alpha-hydroxylase deficiency, 17-alpha-hydroxylase/17,20-lyase deficiency, pseudohermaphroditism, and adrenal hyperplasia.

Table 2: *continued*

Gene	Name	Description
CYP21A2	cytochrome P450, family 21, subfamily A, polypeptide 2	This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and hydroxylates steroids at the 21 position. Its activity is required for the synthesis of steroid hormones including cortisol and aldosterone. Mutations in this gene cause congenital adrenal hyperplasia. A related pseudogene is located near this gene; gene conversion events involving the functional gene and the pseudogene are thought account for many cases of steroid 21-hydroxylase deficiency.
CYP11B1	cytochrome P450, family 11, subfamily B, polypeptide 1	This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the mitochondrial inner membrane and is involved in the conversion of progesterone to cortisol in the adrenal cortex. Mutations in this gene cause congenital adrenal hyperplasia due to 11-beta-hydroxylase deficiency. Transcript variants encoding different isoforms have been noted for this gene.
NR4A2	nuclear receptor subfamily 4, group A, member 2	This gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. The encoded protein may act as a transcription factor. Mutations in this gene have been associated with disorders related to dopaminergic dysfunction, including Parkinson disease, schizophernia, and manic depression. Misregulation of this gene may be associated with rheumatoid arthritis. Four transcript variants encoding four distinct isoforms have been identified for this gene. Additional alternate splice variants may exist, but their full length nature has not been determined.

Table 2: *continued*

Gene	Name	Description
CRH	corticotropin releasing hormone	Corticotropin-releasing hormone (CRH) is a 41-amino acid peptide derived from a 191-amino acid prepro-hormone. CRH is secreted by the paraventricular nucleus (PVN) of the hypothalamus in response to stress. Marked reduction in CRH has been observed in association with Alzheimer disease and autosomal recessive hypothalamic corticotropin dificiency has multiple and potentially fatal metabolic consequences including hypoglycemia and hepatitis. In addition to production in the hypothalamus, CRH is also synthesized in peripheral tissues, such as T lymphocytes and is highly expressed in the placenta. In the placenta CRH is a marker that determines the length of gestation and the timing of parturition and delivery. A rapid increase in circulating levels of CRH occurs at the onset of parturition, suggesting that, in addition to its metabolic functions, CRH may act as a trigger for parturition.
CRHR1	corticotropin releasing hormone receptor 1	The corticotropin-releasing hormone receptor binds to corticotropin-releasing hormone (MIM 122560), a potent mediator of endocrine, autonomic, behavioral, and immune responses to stress.[supplied by OMIM]
POMC	proopiomelanocortin (adrenocorticotropin/ beta-lipotropin/ alpha-melanocyte stimulating hormone/ beta-melanocyte stimulating hormone/ beta-endorphin)	This gene encodes a polypeptide hormone precursor that undergoes extensive, tissue-specific, post-translational processing via cleavage by subtilisin-like enzymes known as prohormone convertases. There are eight potential cleavage sites within the polypeptide precursor and, depending on tissue type and the available convertases, processing may yield as many as ten biologically active peptides involved in diverse cellular functions. The encoded protein is synthesized mainly in corticotroph cells of the anterior pituitary where four cleavage sites are used; adrenocorticotrophin, essential for normal steroidogenesis and the maintenance of normal adrenal weight, and lipotropin beta are the major end products. In other tissues, including the hypothalamus, placenta, and epithelium, all cleavage sites may be used, giving rise to peptides with roles in pain and energy homeostasis, melanocyte stimulation, and immune modulation.

Table 2: *continued*

Gene	Name	Description
MC2R	melanocortin 2 receptor (adrenocorti-cotropic hormone)	MC2R encodes one member of the five-member G-protein associated melanocortin receptor family. Melanocortins (melanocyte-stimulating hormones and adrenocorticotropic hormone) are peptides derived from pro-opiomelanocortin (POMC). MC2R is selectively activated by adrenocorticotropic hormone, whereas the other four melanocortin receptors recognize a variety of melanocortin ligands. Mutations in MC2R can result in familial glucocorticoid deficiency.
CREB1	cAMP-response element-binding protein-1	This gene encodes a transcription factor that is a member of the leucine zipper family of DNA binding proteins. This protein binds as a homodimer to the cAMP-responsive element, an octameric palindrome. The protein is phosphorylated by several protein kinases, and induces transcription of genes in response to hormonal stimulation of the cAMP pathway. Alternate splicing of this gene results in two transcript variants encoding different isoforms.
CREM	cAMP responsive element modulator	This gene encodes a bZIP transcription factor that binds to the cAMP responsive element found in many viral and cellular promoters. It is an important component of cAMP-mediated signal transduction during the spermatogenetic cycle, as well as other complex processes. Alternative promoter and translation initiation site usage allows this gene to exert spatial and temporal specificity to cAMP responsiveness. Multiple alternatively spliced transcript variants encoding several different isoforms have been found for this gene, with some of them functioning as activators and some as repressors of transcription.
SP1	Sp1 transcription factor	Profound changes in patterns of gene expression can result from relatively small changes in the concentrations of sequence-specific transcription factors. Synergistic interaction between factors bound to different sites within a transcriptional control region is supported by the work of Courey et al. (1989). The transcription factor Sp1 is a DNA-binding protein which interacts with a variety of gene promoters containing GC-box elements. Kadonaga et al. (1987) cloned the human Sp1 cDNA and showed that it has contiguous zinc finger motifs and requires zinc for sequence-specific binding to DNA.

Table 2: *continued*

Gene	Name	Description
NR5A1	nuclear receptor subfamily 5, group A, member 1	Studies in adrenocortical cells implicated an orphan nuclear receptor, alternatively designated steroidogenic factor-1 (SF1) or adrenal 4-binding protein (AD4BP), in the gene regulation of the 3 enzymes that are required for the biosynthesis of corticosteroids: cholesterol side chain cleavage enzyme (CYP11A1; 118485), steroid 21-hydroxylase (201910), and the aldosterone synthase isozyme of steroid 11-beta-hydroxylase (CYP11B2; 124080).
NONO	non-POU domain containing, octamer-binding	The SFPQ-NONO-NR5A1/SF-1 complex binds to the CYP17 promoter and regulates basal and cAMP-dependent transcriptional avtivity. NONO binds to an enhancer element in long terminal repeats of endogenous intracisternal A particles (IAPs) and activates transcription (By similarity)
SFPQ	splicing factor proline/glutamine-rich	DNA- and RNA binding protein, involved in several nuclear processes. Essential pre-mRNA splicing factor required early in spliceosome formation and for splicing catalytic step II, probably as an heteromer with NONO. Binds to pre-mRNA in spliceosome C complex, and specifically binds to intronic polypyrimidine tracts. Interacts with U5 snRNA, probably by binding to a purine-rich sequence located on the 3' side of U5 snRNA stem 1b. May be involved in a pre-mRNA coupled splicing and polyadenylation process as component of a snRNP-free complex with SNRPA/U1A. The SFPQ-NONO heteromer associated with MATR3 may play a role in nuclear retention of defective RNAs. SFPQ may be involved in homologous DNA pairing; in vitro, promotes the invasion of ssDNA between a duplex DNA and produces a D-loop formation. The SFPQ-NONO heteromer may be involved in DNA unwinding by modulating the function of topoisomerase I/TOP1; in vitro, stimulates dissociation of TOP1 from DNA after cleavage.

Table 2: *continued*

Gene	Name	Description
SAP130	Sin3A-associated protein, 130kDa	Acts as a transcriptional repressor. May function in the assembly and/or enzymatic activity of the mSin3A corepressor complex or in mediating interactions between the complex and other regulatory complexes. SAP130 is a subunit of the histone deacetylase (see HDAC1; MIM 601241)-dependent SIN3A (MIM 607776) corepressor complex (Fleischer et al., 2003)
MSX2	msh homeobox 2	This gene encodes a member of the muscle segment homeobox gene family. The encoded protein is a transcriptional repressor whose normal activity may establish a balance between survival and apoptosis of neural crest-derived cells required for proper craniofacial morphogenesis. The encoded protein may also have a role in promoting cell growth under certain conditions and may be an important target for the RAS signaling pathways.
NR2F1	nuclear receptor subfamily 2, group F, member 1	Coup (chicken ovalbumin upstream promoter) transcription factor binds to the ovalbumin promoter and, in conjunction with another protein (S300-II) stimulates initiation of transcription. Binds to both direct repeats and palindromes of the 5'-AGGTCA-3' motif.
NR0B1	nuclear receptor subfamily 0, group B, member 1	Orphan nuclear receptor. Component of a cascade required for the development of the hypothalamic-pituitary-adrenal-gonadal axis. Acts as a coregulatory protein that inhibits the transcriptional activity of other nuclear receptors through heterodimeric interactions. May also have a role in the development of the embryo and in the maintenance of embryonic stem cell pluripotency (By similarity). Shuttles between cytoplasm and nucleus.
STAR	steroidogenic acute regulatory protein	The protein encoded by this gene plays a key role in the acute regulation of steroid hormone synthesis by enhancing the conversion of cholesterol into pregnenolone. This protein permits the cleavage of cholesterol into pregnenolone by mediating the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Mutations in this gene are a cause of congenital lipoid adrenal hyperplasia (CLAH), also called lipoid CAH. A pseudogene of this gene is located on chromosome 13.

Table 2: *continued*

Gene	Name	Description
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	The protein encoded by this gene is a member of the SWI/SNF family of proteins and is similar to the brahma protein of Drosophila. Members of this family have helicase and ATPase activities and are thought to regulate transcription of certain genes by altering the chromatin structure around those genes. The encoded protein is part of the large ATP-dependent chromatin remodeling complex SNF/SWI, which is required for transcriptional activation of genes normally repressed by chromatin.
HDAC2	histone deacetylase 2	This gene product belongs to the histone deacetylase family. Histone deacetylases act via the formation of large multiprotein complexes and are responsible for the deacetylation of lysine residues on the N-terminal region of the core histones (H2A, H2B, H3 and H4). This protein also forms transcriptional repressor complexes by associating with many different proteins, including YY1, a mammalian zinc-finger transcription factor. Thus it plays an important role in transcriptional regulation, cell cycle progression and developmental events.
PCSK1	proprotein convertase subtilisin/kexin type 1	The protein encoded by this gene belongs to the subtilisin-like proprotein convertase family. The members of this family are proprotein convertases that process latent precursor proteins into their biologically active products. This encoded protein is a type I proinsulin-processing enzyme that plays a key role in regulating insulin biosynthesis. It is also known to cleave proopiomelanocortin, prorenin, proenkephalin, prodynorphin, prosomatostatin and progastrin. Mutations in this gene are thought to cause obesity.
PCSK2	proprotein convertase subtilisin/kexin type 2	The protein encoded by this gene belongs to the subtilisin-like proprotein convertase family. The members of this family are proprotein convertases that process latent precursor proteins into their biologically active products. This encoded protein is a proinsulin-processing enzyme that plays a key role in regulating insulin biosynthesis. It is also known to cleave proopiomelanocortin, proenkephalin, prodynorphin and proluteinizing-hormone-releasing hormone.

Table 2: *continued*

Gene	Name	Description
MRAP	melanocortin 2 receptor accessory protein	Required for MC2R expression in certain cell types, suggesting that it is involved in the processing, trafficking or function of MC2R. May be involved in the intracellular trafficking pathways in adipocyte cells.

References

[1] M.M. Pollack, K.M. Patel, and U.E. Ruttimann. PRISM III: An updated Pediatric Risk of Mortality score. *Crit Care Med*, 24:743–752, 1996.

[2] R Waterhouse. A case of suprarenal apoplexy. *Lancet*, 1:577–578, 1911.

[3] Del Ray A Besedovsky HO. Immune-neuro-endocrine interactions: facts and hypotheses. *Endocrinol Rev*, 17:64–102, 1996.

[4] S. Reichlin. Neuroendocrine-immune interactions. *N Engl J Med*, 329(17):1246–53, 1993. 0028-4793 (Print) Journal Article Review.

[5] S. Rivest. How circulating cytokines trigger the neural circuits that control the hypothalamic-pituitary-adrenal axis. *Psychoneuroendocrinology*, 26(8):761–88, 2001. 0306-4530 (Print) Journal Article Review.

[6] J. I. Webster, L. Tonelli, and E. M. Sternberg. Neuroendocrine regulation of immunity. *Annu Rev Immunol*, 20:125–63, 2002. 0732-0582 (Print) Journal Article.

[7] G. P. Chrousos. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. *N Engl J Med*, 332(20):1351–62, 1995. 0028-4793 (Print) Journal Article Review.

[8] A. V. Turnbull and C. L. Rivier. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiol Rev*, 79(1):1–71, 1999. 0031-9333 (Print) Journal Article Review.

[9] K. E. Bethin, S. K. Vogt, and L. J. Muglia. Interleukin-6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation. *Proc Natl Acad Sci U S A*, 97(16):9317–22, 2000. 0027-8424 (Print) Journal Article.

[10] M. S. Cooper and P. M. Stewart. Corticosteroid insufficiency in acutely ill patients. *N Engl J Med*, 348(8):727–34, 2003. 1533-4406 (Electronic) Journal Article Review.

[11] T Ganz, ME Selsted, and RI Lehrer. Defensins. *Eur J Haematol*, 44:1–8, 1990.

[12] S Solomon, J Hy, Q Zhy, and et al. Corticostatic peptides. *J Steroid Biochem Mol Biol*, 40:391–398, 1991.

[13] A. V. Panyutich, E. A. Panyutich, V. A. Krapivin, E. A. Baturevich, and T. Ganz. Plasma defensin concentrations are elevated in patients with septicemia or bacterial meningitis. *J Lab Clin Med*, 122(2):202–7, 1993. 0022-2143 (Print) Journal Article.

[14] R. D. Catalano, V. Parameswaran, J. Ramachandran, and D. D. Trunkey. Mechanisms of adrenocortical depression during escherichia coli shock. *Arch Surg*, 119(2):145–50, 1984. 0004-0010 (Print) Journal Article.

[15] P. Liakos, D. Lenz, R. Bernhardt, J. J. Feige, and G. Defaye. Transforming growth factor beta1 inhibits aldosterone and cortisol production in the human adrenocortical cell line nci-h295r through inhibition of cyp11b1 and cyp11b2 expression. *J Endocrinol*, 176(1):69–82, 2003. 0022-0795 (Print) Journal Article.

[16] A. Beishuizen, L. G. Thijs, C. Haanen, and I. Vermes. Macrophage migration inhibitory factor and hypothalamo-pituitary-adrenal function during critical illness. *J Clin Endocrinol Metab*, 86(6):2811–6, 2001. 0021-972X (Print) Journal Article.

[17] M. Jaattela, V. Ilvesmaki, R. Voutilainen, U. H. Stenman, and E. Saksela. Tumor necrosis factor as a potent inhibitor of adrenocorticotropin-induced cortisol production and steroidogenic p450 enzyme gene expression in cultured human fetal adrenal cells. *Endocrinology*, 128(1):623–9, 1991. 0013-7227 (Print) Journal Article.

[18] Hadassah Medical Organization, European Society of Intensive Care Medicine, International Sepsis Forum, The Gorham Foundation. Corticosteroid therapy of septic shock – Corticus.

[19] D Annane and E Bellissante. Corticosteroids for patients with septic shock. *JAMA*, 289:43–44, 2003.

[20] A. H. Hamrahian, T. S. Oseni, and B. M. Arafah. Measurements of serum free cortisol in critically ill patients. *N Engl J Med*, 350(16):1629–38, 2004. 1533-4406 (Electronic) Journal Article.

[21] L. Loriax. Glucocorticoid therapy in the intensive care unit. *N Engl J Med*, 350:1601–1602, 2004.

[22] J. L. Coolens, H. Van Baelen, and W. Heyns. Clinical use of unbound plasma cortisol as calculated from total cortisol and corticosteroid-binding globulin. *J Steroid Biochem*, 26(2):197–202, 1987. 0022-4731 (Print) Journal Article.

[23] Rodbard D Dunn J, Nisul J. Transport of steroid hormones: binding of 21endogenous steroids to both testosterone-binding globulin and corticosteroidsd-binding globulin in human plasma. *J Clin Endocrinol Metab*, 53:58–68, 1981.

[24] U. W. Mueller and J. M. Potter. Binding of cortisol to human albumin and serum: the effect of protein concentration. *Biochem Pharmacol*, 30(7):727–33, 1981. 0006-2952 (Print) Journal Article.

[25] J. T. Ho, H. Al-Musalhi, M. J. Chapman, T. Quach, P. D. Thomas, C. J. Bagley, J. G. Lewis, and D. J. Torpy. Septic shock and sepsis: a comparison of total and free plasma cortisol levels. *J Clin Endocrinol Metab*, 91(1):105–14, 2006. 0021-972X (Print) Clinical Trial Journal Article.

[26] G. L. Hammond, C. L. Smith, N. A. Paterson, and W. J. Sibbald. A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. *J Clin Endocrinol Metab*, 71(1):34–9, 1990. 0021-972X (Print) Journal Article.

[27] Milgrom E. Robin P, Predine J. Assay of unbound cortisol in plasma. *J Clin Endocrinol Metab*, 46:277–283, 1977.

[28] D. Geiseler and M. Ritter. On the validity of free hormone measurements. *Anal Biochem*, 132(1):174–82, 1983. 0003-2697 (Print) Journal Article.

[29] E. G. Lentjes, F. Romijn, R. J. Maassen, L. de Graaf, P. Gautier, and A. J. Moolenaar. Free cortisol in serum assayed by temperature-controlled ultrafiltration before fluorescence polarization immunoassay. *Clin Chem*, 39(12):2518–21, 1993. 0009-9147 (Print) Journal Article.

[30] M. Vogeser, P. Mohnle, and J. Briegel. Free serum cortisol: quantification applying equilibrium dialysis or ultrafiltration and an automated immunoassay system. *Clin Chem Lab Med*, 45(4):521–5, 2007. 1434-6621 (Print) Journal Article.

[31] M Bartels, M Van den Berg, F Sluyter, D I Boomsma, and E J C de Geus. Heritability of cortisol levels: review and simultaneous analysis of twin studies. *Psychoneuroendocrinology*, 28(2):121–37, Feb 2003.

[32] A J Clark and A Weber. Adrenocorticotropin insensitivity syndromes. *Endocr Rev*, 19(6):828–43, Dec 1998.

[33] Hans-Jörg Linde, Joachim Hahn, Ernst Holler, Udo Reischl, and Norbert Lehn. Septicemia due to acinetobacter junii. *J Clin Microbiol*, 40(7):2696–7, Jul 2002.

[34] Stefan Wüst, Elisabeth F C Van Rossum, Ilona S Federenko, Jan W Koper, Robert Kumsta, and Dirk H Hellhammer. Common polymorphisms in the glucocorticoid receptor gene are associated with adrenocortical responses to psychosocial stress. *J Clin Endocrinol Metab*, 89(2):565–73, Feb 2004.

[35] B. M. Arafah. Hypothalamic pituitary adrenal function during critical illness: Limitations of current assessment methods. *J Clin Endocrinol Metab*, 91:3725–3745, 2006. 0021-972X (Print) Journal article.

[36] B. Goldstein, B. Giroir, and A Randolph. International pediatric sepsis consensus conference: Definitions for sepsis and organ dysfunction in pediatrics. *Pediatr Crit Care Med*, 6:2–8, 2005.

[37] J.M. Bland and D.G. Altman. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*, 81(8476):307–10, 1986.

[38] William Y S Wang, Bryan J Barratt, David G Clayton, and John A Todd. Genome-wide association studies: theoretical and practical concerns. *Nat Rev Genet*, 6(2):109–18, Feb 2005.

[39] Christopher S Carlson, Michael A Eberle, Mark J Rieder, Qian Yi, Leonid Kruglyak, and Deborah A Nickerson. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet*, 74(1):106–20, Jan 2004.

[40] Richard Shen, Jian-Bing Fan, Derek Campbell, Weihua Chang, Jing Chen, Dennis Doucet, Jo Yeakley, Marina Bibikova, Eliza Wickham Garcia, Celeste McBride, Frank Steemers, Francisco Garcia, Bahram G Kermani, Kevin Gunderson, and Arnold Oliphant. High-throughput snp genotyping on universal bead arrays. *Mutat Res*, 573(1-2):70–82, Jun 2005.

[41] Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature*, 447(7145):661–78, Jun 2007.

[42] David G Clayton, Neil M Walker, Deborah J Smyth, Rebecca Pask, Jason D Cooper, Lisa M Maier, Luc J Smink, Alex C Lam, Nigel R Ovington, Helen E Stevens, Sarah Nutland, Joanna M M Howson, Malek Faham, Martin Moorhead, Hywel B Jones, Matthew Falkowski, Paul Hardenbol, Thomas D Willis, and John A Todd. Population structure, differential bias and genomic control in a large-scale, case-control association study. *Nat Genet*, 37(11):1243–6, Nov 2005.

[43] Manisha Desai and Mary J Emond. A new mixture model approach to analyzing allelic-loss data using bayes factors. *BMC Bioinformatics*, 5:182, Nov 2004.

[44] M Emond. Power boosting in genome-wide studies via methods for multivariate outcomes. Technical report, UW Biostatistics Working Paper, 2007.

[45] Chris Wallace, Juliet M Chapman, and David G Clayton. Improved power offered by a score test for linkage disequilibrium mapping of quantitative-trait loci by selective genotyping. *Am J Hum Genet*, 78(3):498–504, Mar 2006.

[46] S Greenland. Modeling and variable selection in epidemiologic analysis. *Amer J Pub Health*, 79(3):340–9, Mar 1989.