Midpoint Evaluation: RNAseq Networkwide Project

(a) Cover Page

Co-Principal Investigators:
Kathleen M. Giacomini, University of California, San Francisco
Scott T. Weiss, Channing Laboratory, Harvard Medical School

Investigators:
Steven Brenner, University of California, Berkeley
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Al George, Vanderbilt University
Julie Johnson, University of Florida
Ron Krauss, Children's Hospital of Oakland Research Institute
Deanna Kroetz, University of California, San Francisco
Taimour Langaee, University of Florida
Joshua Lewis, University of Maryland
Marissa Wong Medina, Children’s Hospital of Oakland Research Institute
Audrey Papp, Ohio State University
Wolfgang Sadee, Ohio State University
Steve Scherer, Baylor Sequencing Center
Erin Schuetz, St. Jude's Children's Research Hospital
Alan Shuldiner, University of Maryland
Kelan Tantisera, Channing Laboratory, Harvard Medical School
Leiwei Wang, Mayo Clinic
Dick Weinshilboum, Mayo Clinic
Sook Wah Yee, University of California, San Francisco
(b) Budget

i. Categorical breakdown – See page 8.


iii. Budget Justification

Personnel

Project Director: The PGRNwide RNAseq project is a complex collaboration among eleven Centers in the PGRN; two Network Resources and two working groups. The project involves monthly conference calls among representatives from these groups; transfer of samples between the Centers and the Deep Sequencing Resource(s) (DSRs); potential IRB approvals; transfer of data from the DSRs to the Institute for Genomics and Systems Biology at University of Chicago; posting of data for sharing; data analyses performed at multiple sites; and preparation of multiple publications. The complexity of a project like this requires a Project Director, who serving under the PI (Kathy Giacomini) will devote 50% time to this effort. The Project Director will be responsible for arranging conference calls; assuring that standard operating procedures are developed for RNA quality, data transfer, ensuring that samples are transferred, setting time lines, monitoring progress and facilitating communication among the investigators in the network Centers, the DSRs, and the data sharing site at University of Chicago. Funds are requested for 50% of a Project Director.

Kathy Giacomini, Ph.D, Principal Investigator: Dr. Kathy Giacomini will devote about 1% of her time to the project. Her role will be directing the project, with Scott Weiss, and assuring that progress is being made. This progress includes monthly phone calls, transfer of samples from network centers to DSRs, transfer of data from DSRs to centers and to the University of Chicago database. Kathy Giacomini will supervise the Project Director for the proposed studies.

Consortium and Contractual Costs

RNAseq Funds for DSR: The complete budget justification was included in the original Baylor Subcontract; however, here we provide an update and summary. The subcontract is based on 20M reads per sample and 10x multiplexing per HiSeq lane would come to ~$1000/sample. That price includes sample prep, barcoded library prep, sequencing, data deconvolution, personnel, machine amortization and maintenance. There will be a 1:1 match. That is new funds will be matched with current funds. Directs to Baylor: $147K in Year 3, and $149K in Year 4, plus matching funds from Baylor. These funds will allow us to do about 300 samples per year.

Database/Storage Fund Requests: Institute for Genomics and Systems Biology (IGSB) at the University of Chicago will be involved in data storage. Their justification is included in the subcontracts. In brief, U Chicago requests funding for 6 months of an FTE to enable us to conduct analyses to identify and characterize eQTLs using these data. We will also develop and maintain an analytic pipeline and a public database that can be queried by location, by gene and by SNP, similar to the SCAN database (http://www.scandb.org) that has been widely used in the scientific community. Directs to University of Chicago: $30,000.

XGEN: The XGEN core lab at the Ohio State University will do studies on the CNS samples. Their budget justification is included in attached subcontract and includes RNAseq and genomic sequencing costs as well as bioinformatics support. Directs to Ohio State: $120,000.

UCSF Data Network Charge

This recharge will provide for the support of the campus IT network and was instituted November 1, 2009. Per review and agreement by UCSF’s cognizant federal agency, UCSF data network costs are an allowable direct expense.

Publication Fees

Funds are requested to cover the cost of publication fees, including for Open Access in compliance with NIH and UCSF mandates, for manuscripts that result from these studies.
Summary of Plans From Original Proposal

1. **Very brief background, summarizing the opportunity**

   This large-scale, PGRN-wide hypothesis-generating proposal is focused on RNAseq of tissues, phenotypes and genes of interest in pharmacogenomics research. RNAseq using next-generation technologies most readily provides information about transcript abundance and splice variants, and will be used in the proposed studies to determine expression levels of very important pharmacogenes, VIP genes, and other genes in rich sample sets obtained by PGRN investigators. The proposal leverages the rich tissue and phenotypic resources of the PGRN, the technologies and expertise of the Deep Sequencing Resources (DSRs), the statistical expertise of P-STAR, and the databases developed at the University of Chicago. Several PGRN working groups: the CNS Working Group and the Systems Biology Working Group are involved in the analyses.

2. **Recap of specific aims**

   The specific aims of this hypothesis generating research are as follows:
   
   1. Create a large sharable database and publish a manuscript with quantitative information on gene expression and common splice variants in major organs of pharmacologic interest. This database will be a great resource for the pharmacogenomic community. Focus of the manuscript and database will be on very important pharmacogenes (VIP genes).
   2. Determine the relationship between expression levels of genes and eSNPs identified in lymphoblastoid cell lines and matched tissue samples. *(We decided not to focus on this aim as many eQTL projects have been published; instead we focused on Aims 1 and 3).*
   3. Perform integrative genomic studies linking RNAseq data with and pharmacogenomic phenotypes. These studies may also include GWAS information.

3. **Recap of analysis plans**

   For aim 1, we will simply report expression levels and common splice variants of VIP genes. For this aim our primary analysis will be to map reads, analyze junctures, and determine expression levels of transcripts. We will employ the “tuxedo tools” pipeline of Bowtie, TopHat and Cufflinks to perform mapping, junction discovery, and transcript reconstruction. Abundances of individual isoforms will be quantified using the now-standard FPKM measure. In addition, we will use our JuncBASE pipeline to specifically focus on variations in splicing events between different samples. For aim 2, in addition to the analysis conducted for aim 1, we will perform eQTL analysis. The GGtools package in Bioconductor *(www.bioconductor.org)* will be used to perform the eQTL analysis, which will combine the expression response to drugs to SNPs from GWAS, for example. These analyses will be done for each drug individually. Results will be compared across drugs to see what shared eQTLs exist. For aim 3, in addition to analyses performed in aims 1 and 2, we will perform logistic modeling, which will follow standard epidemiological model-building strategies (e.g. stepwise selection) and Bayes network analyses using the software package, Bayesware Discoverer *(http://bayesware.com)*. This and other machine learning software packages will be used to build coexpression gene networks to further integrate functional QTL expression, and clinical phenotypes. The statistical analysis will be performed by the participating PGRN nodes, with assistance and support of PSTAR and the Systems Biology working group.

4. **Recap of timeline**

<table>
<thead>
<tr>
<th>Year 1 and 2</th>
<th>Complete Aim 1 including data analyses, storage of data in a database, and drafting of manuscript; begin RNAseq experiments of Aims 2 and 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year 3 and 4</td>
<td>Focus on Aims 2 and 3 including analyses of data obtained from 200 RNA samples obtained from each of five PGRN nodes: PHAT, PARC, PEAR, PPII and PAPI</td>
</tr>
</tbody>
</table>

3
(d) Report on Progress to Date

**Aim 1. Create and publish a comprehensive database containing quantitative information on gene expression and splice variants in tissues of pharmacologic interest, with a focus on pharmacogenes.**

To date, the transcriptomes of twenty-five samples from each of four tissues—liver, heart, adipose, and kidney—have been sequenced in order to annotate and quantify the abundance of known and novel isoforms of genes involved in drug response (pharmacogenes). The total RNA was extracted according to an optimized protocol for each research group. After Illumina library protocol optimization, paired-end, strand-specific, poly-A selected libraries were prepared and sequenced on a HiSeq 2000. With the goal of creating a more comprehensive database, RNA-seq data available for other major tissues and cell lines of interest were added to the analysis, including 45 lymphoblastoid cell lines and 14 prefrontal cortex samples. Together, we have initiated analysis of 159 RNA-seq datasets with a computational pipeline that incorporates several available analysis tools, to accurately quantify expression of genes, isoforms, and splice events. Overall, the numbers of genes and transcripts detected were similar across the tissues, indicating no obvious bias. The subsequent steps of the analysis currently underway include (i) transcriptome reconstruction and discovery of novel isoforms using Cufflinks; (ii) gene and isoform abundance quantification and differential expression analysis between and within tissues using Cuffdiff; and (iii) alternative splice event definition and differential expression analysis using JuncBase with a library developed for this project. Preliminary results show that transcript levels of drug metabolizing enzymes, such as MAOA and POR, which are known to be abundant in the liver and kidney are also found in adipose and heart tissues (Figure 1). In addition, multiple known isoforms were identified for each gene, with variability in isoform expression across tissues. A manuscript is being prepared and will include an investigation of differential expression between tissues and of the variability of expression between samples of the same tissue. Complete data will be deposited in dbGaP and/or SRA for subsequent computational and experimental analysis or validation.

![Figure 1. Gene expression levels of 18 selected pharmacogenes in 4 different tissues. Transcripts abundances for each tissue are depicted as (FPKM)$^{1/2}$, with 95% confidence intervals representing the biological variability across five samples from each tissues. In this analysis, the “tuxedo tools” pipeline of Bowtie, TopHat and Cufflinks were used to perform mapping, junction discovery, transcript reconstruction and quantification. These results reveal that some pharmacogenes have large variability in their expression levels between different samples of the same tissues (e.g. CYP2C9, CYP3A4 in the liver) and some genes are more highly expressed in one specific tissue (e.g. cytochrome P450s, NAT2, POR and SLC22A1, in the liver).](image)

**Aim 3. Perform integrative genomic studies linking RNAseq data with and pharmacogenomic phenotypes. These studies may also include GWAS information.**

**A. PARC Project: Identification of transcriptomic variation associated with LDLC (low density lipoprotein cholesterol) response to statin treatment.**

Through our involvement within the PGRN RNA-seq network project, we have generated RNA-seq data from 96 LCLs after in vitro exposure to either simvastatin or sham buffer for 24 hours representing the tails of the in vivo LDLC response distributions in the African American and Caucasian subsets of the CAP population. Indexed, paired-end strand-specific libraries were prepared from polyA-selected RNA and sequenced to a depth of ~80 million, 100bp reads at UW-NEXTGEN, and sequences were aligned to the human transcriptome as well as the human and EBV genomes. Various QC metrics of these data indicated that starting RNA quality, library preparation, and sequencing were all at the highest standard. We identified 21,110 genes in the dataset, more than twice the number detectable by expression array. 6,566 of these genes were not represented on the arrays, while the remaining 3,473 were below the detection threshold. Using a modified paired t-test approach, we found that 3,827 of the 21,100 genes (18%) were changed with statin treatment ($Q<0.0001$). GO term analysis of these genes demonstrated remarkable consistency with those pathways found to be changing with statin response in our original expression array analysis, such that genes involved in sterol biosynthesis and apoptosis up-regulated, while those involved in RNA processing and cell cycle were down-regulated. **These**
findings highlight the superiority of RNA-seq over expression arrays in terms of information obtained, and also demonstrate the consistency of our cellular model system.

To discover statin-induced transcriptome differences between samples derived from “high” versus “low” responders, we first validated sample identity by cross-referencing SNPs found within coding regions of highly transcribed sequences in the RNA-seq dataset with previously obtained genotypic information. In a preliminary analysis of the combined African American and Caucasian samples, transcript X (TX) was the second most differently-expressed transcript and was up-regulated with statin treatment in both the Caucasian and African American high responders, but down-regulated in the low responders of both racial groups (Figure 2A). Notably, TX contains a SNP, S1, in which the minor “G” allele was associated with smaller reductions in LDL-C after statin treatment in a clinical trial. We quantified TX by qPCR in 54 CAP LCLs and found that the minor allele of S1 was associated with reduced TX expression levels after statin treatment (p=0.002), Figure 2B. Additionally, using these same LCLs, which represent participants from the “middle” of the LDLC response distribution, we observed a significant correlation between statin-induced reductions in TX expression levels and reduced total cholesterol lowering after statin treatment in vivo (p=0.05), Figure 2C. These results confirmed our original observation that statin treatment increased TX expression levels in the “high” responders, but decreased expression in the “low” responders, and are consistent with both our findings that the S1 “G” allele is associated with statin-induced reductions of TX expression as well as lower in vivo statin response. These results strongly support the likelihood that TX modulates LDLC response to statin treatment; however, since TX has no known activity, we are now performing functional studies.

![Figure 2](image.png)

Figure 2. Statin-induced change in TX gene expression differs between high and low statin responders. A) TX transcript levels quantified by RNA-seq in Caucasian (CA) and African American (AA) “high” and “low” responders to simvastatin from the CAP clinical trial. B) RP1 transcript levels differ by S1 genotype. C) Correlation between statin-induced change in TX transcript levels quantified in vitro with statin-induced change in total cholesterol quantified in vivo.

We also are in the process of conducting analyses at the exon and splice junction level. In preliminary analyses we have observed that ~7% of gene clusters have at least one statin-responsive exon, independent of gene level changes. GO term analysis showed an enrichment of genes involved in RNA processing and translation, cell cycle, response to stress and sterol/cholesterol metabolism. Similarly, we also found that 1.3% of genes had at least one statin-responsive splice junction. Overall, these results strongly support our ability to use RNA-seq analysis of CAP LCLs to identify novel molecular mechanisms underlying inter-individual variation in statin efficacy.

B. CNS Working Group: RNA-Seq, Central Nervous System

The goal of the CNS-XGen RNA sequencing sub-award is to evaluate the complexity and regulation of RNA expression in the different regions of the human brain, and to assess the effects of chronic nicotine exposure on this gene expression. To date, the Xgen sequencing lab has fully sequenced the long RNA transcripts in nine brain regions in 5 individuals including 3 confirmed smokers and 2 non-smokers. The RNA libraries were sequenced on a SOLiD 5500XL instrument. In these samples, cDNA is prepared using the NuGen Ovation RNA Seq kit™, using 10 ng of total RNA as input material. The NuGen process reduces ribosomal RNA to ~5%, giving good coverage of RNA transcripts. Preliminary expression data on selected genes from the nine regions in these 5 samples are presented in Figure 3. In this small sample set, these genes show trends towards differences in gene expression associated with smoking, in specific brain regions. The preliminary
gene expression results have been distributed to the members of the CNS working group via a private shared network for further analysis. Transcriptome sequencing of the remaining five samples is ongoing. A paper has been accepted for publication, which describes the expression of mRNA transcripts encoding membrane transporters in human brain and liver using RNA-Seq (Webb A et al. Pharmacogen.Genom. (2013) in press).

C. PHAT Project: Identification of transcriptomic variation associated with steroid response in asthma.
PHAT recently received its first set of data and the initial results will be presented at the PGRN spring meeting.

(e) Plans for the Future:
For technical reasons and the need for complex data analytical methodologies, RNAseq remains an enormous challenge for researchers in the biological sciences. Off-the-shelf methods are not readily available to handle the large datasets generated through the sequencing. One of the major strengths of the PGRN Networkwide RNAseq project is that collectively, we are able to solve the immense technical and data analysis problems inherent in RNAseq studies. An example of solving technical issues is that we developed standard operating procedures, SOPs, which allowed us to standardize procedures for RNA extraction, storage and shipping and for RNA quality. These procedures greatly facilitated the RNA sequencing performed at Baylor, which resulted in high quality reads. For data analysis, we appointed a team for Aim 1. The team includes bioinformaticians from the Brenner, Scherer and Cox groups, statisticians from various groups including P-STAR, and biologists/pharmacologists from many groups who understand the importance of the genes to drug response. The team is working efficiently to analyze the data generated in four different tissues of interest. The progress made to date and that will be made in the future is highly dependent on the collective team efforts, which cross nodes of the network and networkwide resources. Below we describe our plans for the future, which will include leveraging the collective skills of the members of the project.

i. Updated specific aims
We have omitted Aim 2 of our previous specific aims. The reasons are that with limited budgets we felt that a focus on the previous Aim 3, integrative studies, would enhance our understanding of the relationships between transcripts and pharmacogenomic phenotypes. In addition, Aim 3 involves LCLs, which was the focus of our previous Aim 2. Below are our modified aims:

**Aim 1.** Create and publish a comprehensive database containing quantitative information on gene expression and splice variants in major organs of pharmacologic interest, with a focus on pharmacogenes.

**Aim 2.** Perform integrative genomic studies linking RNAseq data with and pharmacogenomic phenotypes. These studies may also include GWAS information.

ii. Updated analysis plans, if changes are needed
Our analysis plans are unchanged. Below is a table of the samples available from the network centers participating in the current Aim 2.

<table>
<thead>
<tr>
<th>RNAseq Study Number and Group</th>
<th>Cells and Drugs</th>
<th>Europ. Amer</th>
<th>African Amer</th>
<th>Total Samples</th>
<th>Samples Analyzed in 01 and 02 Years</th>
<th>Samples for Analysis in 03 and 04 Years</th>
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<tbody>
<tr>
<td>1. PARC</td>
<td>Lymphoblastoid Cell Lines Exposed to Statins</td>
<td>609</td>
<td>335</td>
<td>944</td>
<td>200</td>
<td>0</td>
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</table>
2. PHAT  Lymphoblastoid Cell Lines Exposed to Steroids  500  200  700  200  0

3. PEAR  Lymphoblastoid Cell Lines Exposed To Atenolol/ Hydrochlorthiazide  297  194  491  0  200

4. PPII  Lymphoblastoid Cell Lines Exposed to Chemotherapies  400  400  0  200

5. PAPI  Untransformed White Cells and Platelets  300  300  0  200

6. CNS Working Group  10 brain regions from 5 smokers and 5 matched controls  10  10

** iii. Updated timeline for the next 2 years**

**Year 3**
Focus on completion of current Aim 1 and experiments and analyses of the current Aim 2 including analyses of data obtained from 200 RNA samples obtained from each of five PGRN nodes: PHAT, PARC, PEAR, PPII and PAPI. We have performed RNAseq analysis on 200 RNA samples from PHAT and PARC in the current budget year plus 100 samples from Aim 1. In Year 3, we plan to perform RNAseq on 200 samples from PEAR and 100 samples from PPII. In year 3, we anticipate data analysis to be complete on samples from PARC and PHAT. Manuscript for Aim 1 will be completed along with the construction of a publicly available database for Aim 1 data. CNS Plans for Year 3 are: We will complete the RNAseq analysis of all remaining brain regions, using a sample preparation that captures all coding and non-coding RNAs in each tissue. We will also perform whole genome sequencing using an ion torrent technology, as a guide to transcriptome analysis. As the RNAseq preparations do not capture short non-coding RNAs of <200 bases (such as miRs), we will attempt to prepare these samples also for small RNA analysis. Data mining will become increasingly important for extracting valuable information from the growing database, including nicotine effects and interactions between coding and non-coding RNAs.

**Year 4**
We will perform RNAseq on 100 samples from PPII and 200 from PAPI. In addition, we will prepare manuscripts for studies from PHAT and PARC. Data analyses for PPII and PAPI studies will be performed and manuscripts will be prepared. CNS plans for Year 4 are: We will have completed RNAseq analyses on all samples and will begin to assay interesting transcript individually to validate results. If any possible (financially), we will complete analysis of short non-coding RNAs, which may prove critical to a full understanding of expression profiles in different tissues between subjects as a function of nicotine exposure. Genes of interest will also be assayed in additional brain samples (available in the Sadee lab). We further expect several follow-up studies on protein expression by investigators of the CNS group and other PGRN members or outside collaborators.

**iv. Updated data sharing plans**
Data sharing plans are unchanged and are as follows: We are well positioned to build on existing resources and infrastructure (such as our large-scale databases) and to utilize ongoing efforts in a cloud-based data management solution developed by the Institute for Genomics and Systems Biology (IGSB) at the University of Chicago. Data will be transmitted from the sequencing center to IGSB, where the data will be made available to all contributing investigators. After one year, data will be made publicly available. CNS data will be shared among the CNS Working group and then will be made publicly available. CNS data will shared among the CNS Working group and then will be made publicly available. Also, other investigators of the PGRN can request access to the data; we will also transfer datasets to the IGSB under the same rules stated above.

Because of budget constraints, we are only able to analyze 200 samples from each of the participating centers; however each center as noted in Table 1 has many more samples. We are planning on resubmitting a proposal to the DSRs for analysis of additional samples from each of the groups.
<table>
<thead>
<tr>
<th>NAME</th>
<th>ROLE ON PROJECT</th>
<th>Cal. Mnths</th>
<th>Acad. Mnths</th>
<th>Summer Mnths</th>
<th>INST. BASE SALARY</th>
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<th>FRINGE BENEFITS</th>
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<tr>
<td>Kathleen M. Giacomini</td>
<td>PD/PI</td>
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<td>*</td>
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*actual salary exceeds the cap

**SUBTOTALS**

|                        | 51,797 | 16,057 | 67,854 |

**CONSULTANT COSTS**

**EQUIPMENT (Itemize)**

**SUPPLIES (Itemize by category)**

**TRAVEL**

**INPATIENT CARE COSTS**

**OUTPATIENT CARE COSTS**

**ALTERATIONS AND RENOVATIONS (Itemize by category)**

**OTHER EXPENSES (Itemize by category)**

UCSF Data Network 220
Publication Fees 279

CONSORTIUM/CONTRACTUAL COSTS

| DIRECT COSTS | 297,000 |

**SUBTOTAL DIRECT COST FOR YEAR 3 BUDGET PERIOD (Item 7a, Face Page)**

$ 365,353

CONSORTIUM/CONTRACTUAL COSTS FACILITIES AND ADMINISTRATIVE COSTS 122,871

**TOTAL DIRECT COSTS FOR YEAR 3 BUDGET PERIOD**

$ 488,224

PHS 398 (Rev. 6/09)
BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

<table>
<thead>
<tr>
<th>BUDGET CATEGORY</th>
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<th>YEAR 4</th>
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TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD $980,233

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Pursuant to University of California policy, salaries in the initial budget period are based on current published UC salary scales and include University mandated range adjustments and merit increases scheduled to occur before the proposed project start date.