THE CHARM-01 STUDY: ASSESSING FORMULATIONS OF TENOFOVIR 1% GEL IN HIV SERONEGATIVE ADULTS VIA TRANSCRIPTOME ANALYSIS
CHARM-01

- Double-blinded, randomized, safety & acceptability, pharmacokinetic, and ex vivo efficacy study of rectally-applied tenofovir-based microbicide formulations.
- Dose comparison of 3 current formulations of tenofovir 1% gel
  - Vaginal formulation
  - Reduced glycerin formulation
  - Rectal specific formulation
- Endpoints
  - General and mucosal safety
  - Pharmacokinetics and pharmacodynamics
- Current status
  - Completed

McGowan I et al. PLoS ONE 2015
Transcriptomic Study Objective and Aims

**Transcriptome**: The complete set of coding and non-coding transcripts in a given sample and their quantity

**Expanded Objective**: Apply low-input RNA-Seq transcriptional analysis as a sensitive assay to uncover changes to the mucosal environment caused by gel usage.

**Hypothesis**: Local changes in the mucosal immune environment (e.g. IFNs/inflammasome) may be part-and parcel the action of microbicide treatment and alter risk of HIV infection

**Aim1**: To distinguish the effects of three microbicide tenofovir 1% gel formulations, administered rectally, using transcriptomics

**Aim2**: To compare data with other microbicide trials and validate signatures of intestinal mucosal gene expression following microbicide exposure associated with protection or risk
CHARM-01 Study Design
Blinded crossover design with Tenofovir 1% formulations in random sequence

Visit 1: Screening

Endoscopy
with biopsy

Visit 2: Enrollment / Baseline evaluation

Microbicide tenofovir 1% gel formulations:

- **Reduced-Glycerin Vaginal Formulation** (RGVF, 846 mOsmol/kg)
- **Rectal-specific Formulation** (RF, 479 mOsmol/kg)
- **Vaginal Formulation** (VF, 3111 mOsmol/kg)

Each participant will receive:
7 rectal exposures to RGVF
7 rectal exposures to RF
1 exposure to VF, coupled with 6 preceding exposures to Universal HEC Placebo Gel

N = 14 patients
Total RNA was isolated from 50 gut biopsies preserved in RNAlater using Qiagen RNEasy Mini Plus Kits.

RNA-Seq data was generated using Illumina Truseq (low input SOP) kits and the Illumina HiSeq 2500:

- Paired-end, 50 cycle, >30x10^6 mapped reads/sample
- Medium depth: Able to measure the transcriptome and common splicing variants.

RNA-Seq’s advantage areas over microarray include sensitivity, specificity and quantification, as well as fielding coding (mRNA) and non-coding (e.g. small RNA) transcript counts.
Preprocessing of RNA-seq: R Pipeline

1. Create **Raw fastq** from binary output by running pooled tagged RNA short read libraries are run on Illumina Hiseq 2500 (2x50 30M reads), as per manufacturer’s instructions.

2. Create **High Quality Fastq’s** by filtering out poor quality base calls and adapter contamination using **Trimmomatic**.

3. Generate **Mapped Read** files by aligning the HQ Fastq reads using the STAR aligner.

4. Generate **Features Counts** using **Htseq**.
Gene based Analysis

1. Create **Raw Gene Expression Matrix (Genes by Samples)** from aggregating count files into a matrix, and importing the sample Phenotype data in R.

2. Create **Normalized Expression Matrix** by performing removing sample outliers base on QC assessment and normalizing the samples to each other using Edge.

3. Generate **Differential Gene Expression Lists** by performing 2 group analysis via linear modeling using EdgeR.

4. Generate **Pathway Enrichment Lists** by taking top ranking genes and performing pathway enrichment using Gene Set Enrichment Analysis or GSEA.
Effects of three microbicide tenofovir 1% gel formulations on intestinal mucosal gene expression

Comparing formulations to baseline: F-Test

Method: Fitting GLM (GLM: R - EdgeR)
Model: (Gene expression ~ Formulation group + Donor)

Kruskal wallis rank sum test

Dimension 1

<table>
<thead>
<tr>
<th>Group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.002</td>
</tr>
<tr>
<td>RGVF</td>
<td>0.001</td>
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</tbody>
</table>

RGVF and Baseline significantly explain the first dimension of variation.

Multi Dimensional Scaling analysis of the top 500 F-test genes by P value shows RGVF has the most significant expression profile compared to baseline.

Comments: There is a lot of variability between donors (n=12-14) in gene expression observed within the formulation groups.
Solution: Gene Set Enrichment Analysis: RGVF – Baseline Focused

Many increased pathway activities upon exposure to RGVF
Oxidative phosphorylation, mTORC1 signaling, and cell cycle genes are up-regulated upon exposure to RGVF formulation

- Genes encoding proteins involved in oxidative phosphorylation
- Genes encoding cell cycle and related targets of E2F transcription factors
- Genes important for mitotic spindle assembly
- Impact on cell proliferation, stress response

Ph related?

- Activation of the mTORC1 complex
- Impact on senescence and T cell fate
Inflammasome-related pathways are up-regulated upon exposure to RGVF formulation

- Genes up-regulated in response to IFNA
  E.g. OAS1, LAMP3, IFITM1

- Genes up-regulated in response to IFNG
  E.g. CXCL9, SOCS1, GZMA

- General IFN response genes
  E.g. IRF7, CXCL10, IFIs/IFITs, MX1, ISG20

- Genes associated with the complement system
  E.g. SERPINs, FCN1, C2, CASP1/5

- Genes up-regulated during allograft rejection
  E.g. TAP1/2, IL10, IFNG, HLAs
Comparison: Project Gel GSEA: (RG Tenofovir Day1)

Communication Between Innate and Adaptive Immunity

IFNG-mediated pathway signatures do not persist to D7 in Project Gel, however…
Inflammasome signatures are associated with early/late menstrual cycle phases (Gaucher et al., 2008).

CVL Samples at Early Phase

BIRC3: regulates caspase cascades inflammatory gene signaling

Cytobrushes

Early/Proliferative

Late/Secretory

Inflammasome-filtered
Enrichment in B cell and monocyte-related genes describes the D1 and D7 Project Gel gene expression signature

Deconvolution: OMIC Module-Based Filtering
Nakaya et al. (Nat Immunol 12(8): 786, 2011)
Conclusions:

- While the N and effect sizes are small, we have begun to hone a signature associated with rectal application of tenofovir gel.
- RGVF has the most unique expression profile compared to baseline:
  - Increased pathway activities include proinflammatory pathways, IFNs, cell signaling, stress and cell cycle.
  - Coordinated inflammasome involvement in RGVF formulation.
  - Balance between antiviral IFNs and the greater inflammasome is likely delicate in determining an outcome and a result of many factors.
  - Need outcomes to give this balance context in risk.
- CHARM inflammation signature can be seen early in Project Gel:
  - IFNG/IFNG-response genes, SERPINs, SOCS, chemokines are up-regulated.
- Nakaya filtering (a deconvolution method) indicate that gene activity in Project Gel may stem from alterations in B cell and Monocytes/MDC.
A lot of work remains:

Perform additional contrasts to pair an individual subject with their baseline and formulation sequence and map their overall responses in the MDS and inflammasome

- hampered by lower subject numbers and resulting bioinformatic approaches (e.g. ranking DGE by nominal P)

Increase subject numbers, integrate different data types (flow data, proteome) and outcomes, train and test signatures in similar human trials, or meta-analysis across very different studies (human, NHP)

Develop and validate new flow panel and/or Fluidigm PCR panel to probe these signatures at a finely sorted and in titration down to the single cell level

Identify and target mechanisms and biomarkers of protection or infection risk in future trials
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