Effect of E-cigarette Vapor on Hemopoetic Stem Cell Function

Group: Mouse 1
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1 Abstract

While the effects of cigarette smoking on hematopoeietic stem cell (HSC) function have been studied extensively, not much literature exists on the effects of e-cigarette use (vaping). In this experiment, we investigate the effect on HSC's and progenitor cells of ongoing exposure to e-cigarette vapor, as well as the effect on HSC function of *past* exposure to e-cigarette vapor. We find strong indications (if not statistically significant once a multiple-testing correction is applied) that ongoing exposure to e-cig vapor suppresses HSC function, leading to lower numbers of HSC's and progenitor cells. As to the effects of past exposure on HSC's, our findings are inconclusive, though in general consistent with the scientific consensus on vaping, i.e. the fitted model suggests, weakly, that past exposure also suppresses HSC function.

2 Introduction

An electronic cigarette[1], also known as e-cigarette (vaping) is an electronic device that simulates tobacco smoking. Vaping devices are popular among teens and are now the most commonly used form of nicotine among youth in the United States, and early evidence[2] suggests that vaping might serve as an introductory product for preteens and teens who then go on to use other nicotine products, including cigarettes, which are known to cause disease and premature death.

Unfortunately, while there is extensive literature of effects of tobacco smoke on hematologic derangements, there is very little on the effects of e-cigarette use. In this study, we examine whether vaping has similar effects on hematopoietic stem cells as cigarettes. In particular, it has been demonstrated that exposure to smoking is associated with increased risk for Clonal Hematopoiesis of Indeterminate Potential (CHIP), which is an established risk factor for hematologic cancers, heart attacks, stroke. Since nicotine leads to inferior developmental colonization of Bone Marrow by hematopoietic stem cells (HSC), and since HSCs give rise to other blood cells and can proliferate indefinitely, changes in number and function of HSCs are thought to be a main driver in increased risk of CHIP.

Of particular interest is whether the effects of e-cigarette use parallel the relationship between cigarette smoking and Bone Marrow[3]. The direct effect of cigarette smoking on Bone Marrow is supported by experimental studies that reveal a reduction of hematopoietic progenitor cells (HPC) after mice were exposed to cigarette smoking for 3 weeks. Work by Robert W. Siggins et al. in 2014 concluded that cigarette smoke alters the Hematopoietic Stem Cell Niche through changes in the stem cell environment[2].

So far, the effects of e-cigarette (vaping) on hematologic derangements have received little attention. The focus of this study will be on the change of stem cells themselves after exposure to e-cigarette vapor: Does e-cigarette use lead to changes in bone marrow stem and progenitor populations? To study whether vaping induces chronic oxidative stress and inflammation that can affect hematopoietic stem cell number and function leading to CHIP, we exposed thirty mice to e-cigarette vapor (treatment) and air (controls) for

two months; seven mice from the air group and seven from the e-cigarette group were randomly chosen for Bone marrow HSC analysis. In addition, unlike previous studies (Siggins et al [4]), instead of transplanting regular cells into vapor-exposed mice, the vapor-exposed cells were transplanted into normal mice. Of particular interest in this study is the impact of e-cigarette vapor on HSPC (hematopoietic stem and progenitor cells) pool size and HSC function via functional assay, in which the selective pressure induced by exposure to e-cigarette vapor is evident through competition between the treatment- and control-group stem cells themselves and wild-type stem cells.

2.1 Purpose of the Analysis

- **Hypothesis:**
  - Similarly to smoking, vaping is likely to induce chronic oxidative stress and inflammation that can affect HSC population size and function (promoting CHIP).
- **Goals:**
  - establish effect of E-cig vapor via pilot study
  - hypothesis generation for larger trial
  - inform experimental design of larger trial

3 Study Methods

3.1 General Study Design and Plan

The experiment is conducted in two phases: the first phase investigates the effect of *ongoing* exposure to e-cigarette vapor on the HSPC pool in mice; the second phase investigates whether exposure to e-cigarette vapor has a *lasting* effect on HSC function.

3.1.1 Phase 1: effect of ongoing e-cig vapor exposure on HSPC's.

The purpose of this phase is to assess the effect of ongoing exposure to e-cigarette vapor on various types of stem and progenitor cells, by conducting a controlled experiment to examine the frequency and absolute counts of bone marrow HSPCs of between a group of mice exposed to e-cig vapor, and another group exposed to clean air. In particular, we would like to assess the hypothesis on whether e-cig vapor effects the proliferation of stem and progenitor cells (i.e. is there a difference in absolute counts/frequencies of HSPCs between the air/e-cig group).

A total of 30 eight-week-old CD45.2-type mice are randomly assigned into one of either the "air" group or the "e-cig" group, such that 15 mice were in each group. (Note that the fact that these are CD45.2-type mice is not relevant for this first phase; the ability to detect the difference between cells originating from CD45.2 and CD45.1-type mice becomes useful in the next phase of the experiment.) 7 mice from each group are assigned to be placed in one of two respective rooms for each group. Regular air is sent into the room for the air group of rats, while concentrated levels of e-cig vapor are sent off into the room for the e-cig group of rats, with air concentration levels being watched by a Aerosol Mass Monitor. After eight weeks, all of the mice in the experiment were terminated in order to take samples of their bone marrow, from which HSPC counts were recorded.

3.1.2 Phase 2: functional assay of e-cig vapor-exposed HSC's.

The purpose of this phase of the experiment is to assess whether the function of hematopoetic stem cells are compromised by exposure to e-cigarette vapor. We accomplish this by placing the HSCs from both the treatment and control groups in Phase 1 in competition with a new group of stem cells. In particular,

we wish to observe whether e-cig vapor perturbs HSC function even after exposure has ended, and in particular if it has an effect on the ability of HSC's to repopulate a host mouse's leukocyte pool.

Bone marrow is harvested from the CD45.2-type mice in Phase 1, and separated by Phase 1 treatment group. Harvested bone marrow from the e-cig vapor-exposed mice are then mixed together; similarly for the bone marrow from the control group mice. These donor bone marrow cell (BMC) mixtures are then each mixed in a 50-50 ratio with bone marrow from CD45.1 mice that have not been experimented upon, resulting in two mixture preparations: 1 preparation with 50% wild-type CD45.1 bone marrow cells (BMCs) and 50% CD45.2 BMCs from mice exposed to e-cigarette vapor, and 1 preparation containing 50% wild-type CD45.1 BMCs and 50% CD45.2 BMCs from mice that were not exposed to e-cigarette vapor. (We will refer to these as "e-cig donor marrow" and "Air donor marrow"). Another mixture following the same procedure is made for mice which were intended as positive controls, but not held in the same type of holding cages in Phase 1.

Note that CD45.1-type mice and CD45.2-type mice are essentially the same mice; they differ only in that they have differing alleles at a specific gene which does not impact stem cell function but makes it such that investigators are able to distinguish between cells originating from the different types of mice. Thus we are able to distinguish between cells originating from the donor stem cells from phase I (CD45.2) and cells originating from stem cells derived from the new, wild-type mice (CD45.1).

These donor BMC solutions are then transplanted into 15 host mice which have been irradiated to deplete the host's own HSC's. 6 mice chosen at random received the e-cig donor marrow, 6 mice received the air donor marrow, and the remaining 3 mice received the positive control mouse donor marrow. Since the host mice's own HSC's have been depleted via radiation, the stem cells in the donor marrow mixtures (CD45.2 and CD45.1 in 50-50 ratios) will compete to repopulate the host's leukocyte and red blood cell population. The proportion of CD45.2-type leukocytes to both CD45.1 and CD45.2-type leukocytes in blood draws thus indicates whether the function of the CD45.2 HSC's has been compromised by exposure to oxidative stresses from e-cigarette vapor versus air.

Blood draws were conducted every 4 weeks beginning at week 4; frequency of blood draws was increased after week 16 to every 2 weeks. Blood samples from each mouse were analyzed using flow cytometry after staining; CD45.2-type leukocytes (treatment and control) and CD45.1 leukocytes (wild-type competitor) were counted after gating. Gating was performed by the same person to promote uniformity. Host stem cell-derived leukocytes were counted as well, though their numbers were few since the stem cells giving rise to these leukocytes were the small percentage that survived radiation treatment before transplantation of donor marrow.

3.2 Exploratory Data Analysis and Statistical Modeling

3.2.1 Primary Objectives

- **Primary Objectives:**
  - Test whether *ongoing* exposure to E-cigarette vapor impacts HSPC pool size in mice;
  - Investigate the impact of *past* exposure to E-cigarette vapor on HSC function via functional assay.
- **Secondary Objectives:**
  - Perform power analysis based on pilot study results;
  - Assess current study design for impact of past exposure.

3.2.1.1 Experiment Phase 1: In order to figure out whether E-cigarette vapor has influence on the number and function of hematopoietic stem cells before transplant, we propose our null and alternative hypotheses:

H_0: E-cigarette has no effect on the in hematopoietic stem cell and progenitor populations,
H_1: E-cigarette truly has effect on the in hematopoietic stem cell and progenitor populations.

Based on the experts' opinions, we choose five hematopoietic stem and progenitor cells of great research interest from the murine hematopoietic system, including LKS-SLAM, LKS, CMP, MEP and GMP. Therefore, we propose five sub null hypotheses: the percentages of each cell within e-cig vapor-exposed mice and air-exposed mice are the same.

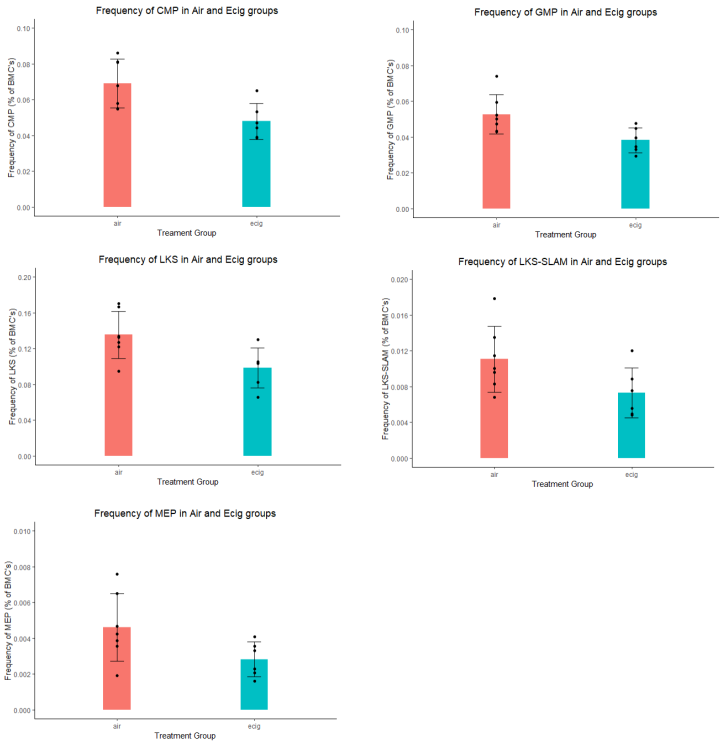

Figure 1: E-cigarette vapor decreases frequency of bone marrow HSPCs

From the bar plots (Figure 1) there is strong visual evidence to suggest that HSPC frequency is lower in the e-cig group. We do note that the standard error is somewhat large for both groups when looking at LKS and LKS-Slam frequencies. To explore the difference further, we use simple t-test to make examinations. After that, the HolmBonferroni method is used to conduct our multiple comparisons, which is intended to control the family-wise error rate under the significant level α and offers a more powerful test with the less conservative result. The following are the steps of Holm–Bonferroni method:
1. Let $H_{01}, ...H_{05}$ be a family of $m = 5$ null hypotheses and $P_1, ..., P_5$ the corresponding p-values.
2. Start by ordering the p-values (from lowest to highest) $P_{(1)}, ..., P_{(5)}$ and let the associated hypotheses be $H_{(1)}, ..., H_{(5)}$.
3. For a given significance level $\alpha = 0.05$, let k be the minimal index such that $P_{(k)} > \frac{\alpha}{m+1-k}$.
4. Reject the null hypotheses $H_{(1)}, ..., H_{(k-1)}$ and do not reject $H_{(k)}, ..., H_{(5)}$.

3.2.1.2 Experiment Phase 2: The counts of CD45.1 and CD45.2 cells from 12 mice (6 Air and 6 E-cig) over 5 observations (week 4,8,12,18,20) are used for analysis. First we explore the varying trend of the proportion of CD45.2 cells of Air and E-cig groups over time. As shown in figure 2, the proportion approximately grows linearly over time. Air group shows larger variation among subjects. Subject 2042

(Air) and 2054 (E-cig) are potential outliers that may require further examination. To see the overall trend of the proportion of CD45.2 cells, we take weighted average trajectories of all subjects within Air and E-cig groups, as shown in figure 3. The average is weighted by the number of cells at each observation, because the sample sizes of cells obtained at each observation varies a lot and was not controlled during experiment, as shown in figure 4. This gives us the motive to use a binomial model.

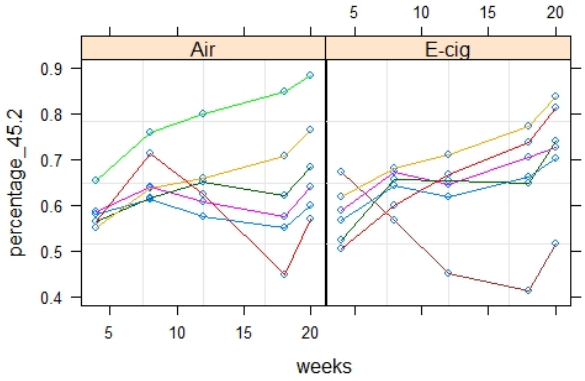

Figure 2: Individual varying trend of the proportion of CD45.2 cells over time.

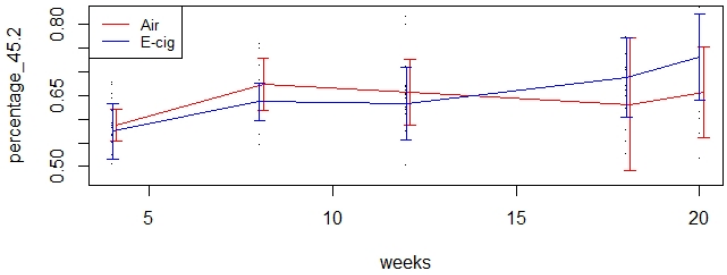

Figure 3: Mean plot of the proportion of CD45.2 cells over time.

Since the data contains longitudinal observations, we then explore the covariance structure of the data. First the time variable is categorized into several periods in convenience for analysis. Residuals of the proportion are obtained by fitting natural cubic splines with respect to periods of time. Figure 5 shows the pairwise residual plots of the proportion over time and their corresponding correlation. The correlations between neighboring time periods are evident, but as time lag grows the relations generally show a decreasing pattern. Variogram in figure 6 obtained from the residuals characterizes the covariance structure. Noticeable serial process is reflected by the increasing trend. So auto-regression structure AR-1 (correlation exponentially decreases over time) is selected as covariance structure for analysis over time.

To answer whether there is a difference in the proportion of CD45.2 cells between Air and E-cig group, we use the generalized linear mixed-effects model (GLMM) model to process binomial and longitudinal data. The `lme4` package in R is used to achieve this object. To apply the GLMM model, both assumptions

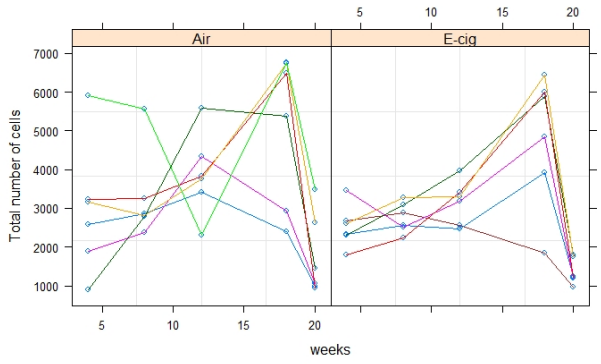

Figure 4: Total number of CD45.1 and CD45.2 cells

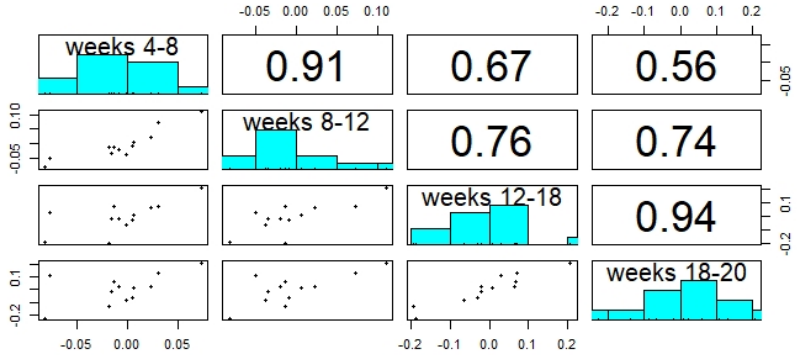

Figure 5: Pairwise residuals of the proportion of CD45.2 cells over time.

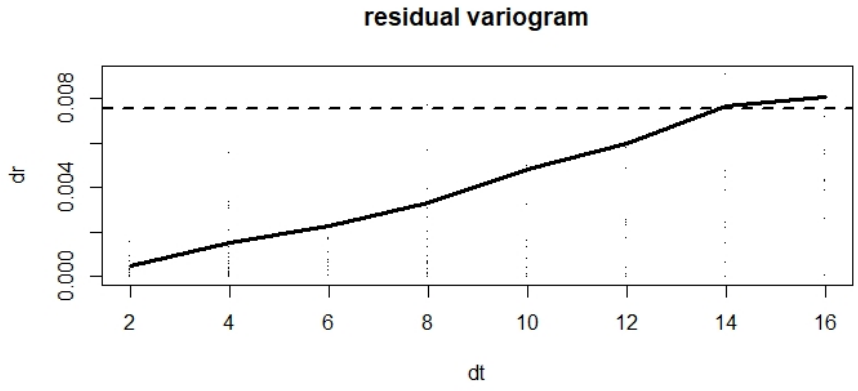

Figure 6: Variogram of the proportion of 45.2 cells over different time periods.

of mean model and covariance structure are required. The mean model is assumed to be as follows:

$$log(\frac{\mu_{ij}}{1-\mu_{ij}}) = \beta_0 + \beta_1 t_{ij} + \beta_2 trt_i$$

where μ_{ij} denotes the expected proportion of CD45.2 cells, t_{ij} the time variable, and trt_i the indicator of

E-cig group in j-th observation for mouse i. Here β_2 is the variable of interest, which can be interpreted as the log-odds ratio of the expected proportion of CD45.2 cells between Air and E-cig groups. The primary null hypothesis for experiment 2 then can be framed as $H_0 : \beta_2 = 0$.

GLMM acquires the probability assumptions on the response distribution. Based on the exploratory data analysis of the covariance structure of the data, we assume a random intercept and random slope to describe the correlation decaying process. Therefore the overall statistical model can be formulated as following:

$$Y_{ij} \sim Bin(\mu_{ij}, m_{ij})$$
$$log(\frac{\mu_{ij}}{1-\mu_{ij}}) = b_{0j} + b_{1j}t_{ij} + \beta_2 trt_i$$
$$\begin{pmatrix} b_{0j} \\ b_{1j} \end{pmatrix} \sim N\left(\begin{pmatrix} \beta_0 \\ \beta_1 \end{pmatrix}, \begin{pmatrix} \sigma_0^2 & \rho\sigma_0\sigma_1 \\ \rho\sigma_0\sigma_1 & \sigma_1^2 \end{pmatrix}\right)$$

where m_{ij} denotes the is the number of cells collected at j-th observation for mouse i.

4 Results

4.1 Experiment Phase 1

We Applied the `t.test()` in R to conduct the two sample t-test and used Holm-Bonferroni Method to deal with familywise error rates for multiple hypothesis tests.

			a=0.05	Holm-Bonferroni	
Primary Outcome	test statistics	P value	Reject H0	alpha level	Reject H0
CMP	3.24	0.008	Yes	0.01	Yes
GMP	2.86	0.016	Yes	0.0125	No
LKS	2.75	0.018	Yes	0.0167	No
MEP	2.21	0.054	Yes	0.025	No
LKS_SLAM	2.08	0.061	No	0.05	No

Table 1: Result of t test under a=0.05 and Holm-Bonferroni Method

As shown in Table1, we could not reject the most of the H_0 under Holm-Bonferroni Method. There is no enough statistical evidence to support that there is a different between E-cigarette and air on GMP, LKS, MEP and LKS_SLAM.

4.2 Experiment Phase 2

The coefficient estimates of the GLMM are listed in the table 4.2. Estimation of β_1 shows that overall the odds of CD45.2 cell proportion has a relatively strong positive relation with time. $\exp(\beta_2)$ is the odds ratio of CD45.2 cell proportion between E-cig and Air groups. From the results in table 4.2 we estimate that the odds that a cell will be CD45.2 will be 0.09 times lower in the E-cig group than in the Air group. At a 95% confidence level, we believe the odds will be between 23% lower to 8% higher, which is fairly inconclusive (not statistically significant).

To illustrate the model fitting, we plot the estimated CD45.2 cell proportion at each observation, shown in figure 7. Since our model assumes a linear relationship with time, the trajectories are nearly straight apart from slight fluctuations due to the effects of random intercepts and random slopes.

	Est	exp(Est)	ci95.lo	ci95.hi	z value	Pr(>|z|)
β_0	0.38	1.46	1.24	1.72	4.49	0.00***
β_1	0.02	1.02	1.00	1.05	1.86	0.06*
$\boldsymbol{\beta_2}$	**-0.09**	**0.91**	**0.77**	**1.08**	**-1.10**	**0.27**

Table 2: Coefficient estimates and their confidence intervals of the GLMM

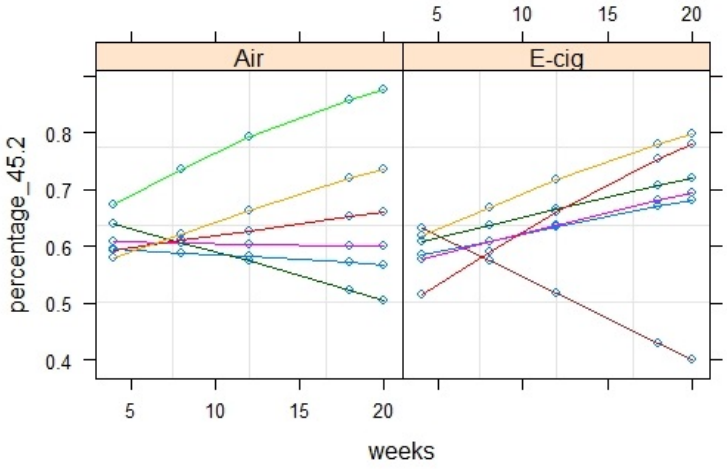

Figure 7: Fitted spaghetti plots of CD45.2 proportions over time.

5 Sample Size & Power Analysis

5.1 Experiment Phase 1

A statistical power analysis was performed for sample size estimation, treating experiment 1 as a pilot study (N = 13) comparing differences in percentages of cells between the e-cig vapor-exposed mice group and air-exposed mice group.

Since the sample size is not too big here, it is reasonable to choose α=0.1, if we choose α=0.01 the standard error of each group will be large. Fixed the α = 0.05 and a=0.1 with power = 0.7,0.8,0.9, the result of estimated sample sizes for the following study are shown below.

Moreover, we set a range for the standardized effect sizes based on what we observed in the pilot data, and make plots of sample size with various combinations of α and power as mentioned above. (See Figure 8-9,)

	Significant level					
	a=0.05			a=0.1		
	Power					
	0.7	0.8	0.9	0.7	0.8	0.9
LKS-SLAM	11	14	18	9	11	14
LKS	7	8	11	5	7	9
CMP	6	7	8	4	5	7
MEP	11	13	17	8	10	14
GMP	7	8	10	5	7	9

Table 3: Result of power analysis under Phases I

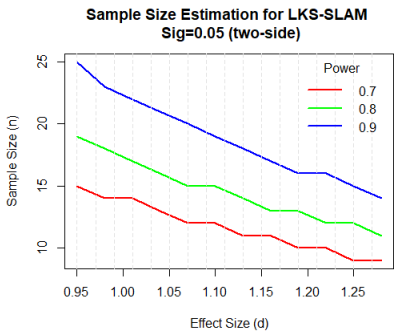

Figure 8: Est. Sample Size (sig=0.05)

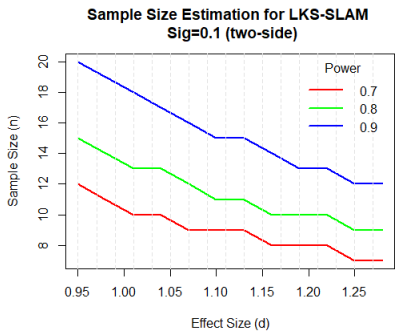

Figure 9: Est. Sample Size (sig=0.1)

As shown as in Table 1, since the LKS-SLAM and LKS are primary variables of our study interests, we suggest that if 14 mice can be applied in e-cigarettes and air group, respectively, in Phase I, the statistical power of 5 tests could all achieved at least 80%. Figure 8 presents the necessary sample size for testing LKS-SLAM with power at 0.7, 0.8 and 0.9, α=0.05 in a range of effect size, we can observe that every 0.1 increase in power, about 5 mice are required to be included into each group.

5.2 Experiment Phase 2

For experiment phase 2, we use simulations to conduct the power analysis. The following procedures are used for data generation:

$$\begin{pmatrix} b_{0j} \\ b_{1j} \end{pmatrix} \sim N\left(\begin{pmatrix} \hat{\beta}_0 \\ \hat{\beta}_1 \end{pmatrix}, \begin{pmatrix} \hat{\sigma_0}^2 & \hat{\rho}\hat{\sigma}_0\hat{\sigma}_1 \\ \hat{\rho}\hat{\sigma}_0\hat{\sigma}_1 & \hat{\sigma_1}^2 \end{pmatrix} \right)$$

$$\mu_{ij} = \frac{exp(b_{0j} + b_{1j}t_{ij} + \beta_2 trt_i)}{1 + exp(b_{0j} + b_{1j}t_{ij} + \beta_2 trt_i)}$$

$$m_{ij} \sim 100 \times Poisson(30)$$

$$Y_{ij} \sim Bin(\mu_{ij}, m_{ij})$$

The procedures are roughly the inverse of the statistical modeling. Except β_2, all parameters are estimated through the real data set (hence the hats). The scaled Poison generation of m_{ij} is based on the distribution of cell counts (figure 4).

To calculate the power for different settings, the following attributes are varied in certain ranges: (1) Significance level α = (0.05, 0.1); (2) Sample size N = (12,16,20,24); (3) Effect size $exp(\beta_2)$ = (0.97,0.94,0.91,0.88,0.85) (decreasing magnitude means increasing effect size). For each setting, 500 groups of data are generated and each fitted by GLMM to estimate the power.

N \ $exp(\beta_2)$	0.97	0.94	0.91	0.88	0.85
12	0.14	0.20	**0.29**	0.41	0.54
16	0.10	0.19	0.31	0.46	0.63
20	0.14	0.21	0.35	0.58	0.72
24	0.12	0.21	0.39	0.61	0.79

Table 4: Estimated power by simulation with $\alpha = 0.05$

N \ $exp(\beta_2)$	0.97	0.94	0.91	0.88	0.85
12	0.20	0.28	**0.37**	0.48	0.66
16	0.17	0.27	0.40	0.58	0.71
20	0.22	0.29	0.47	0.67	0.81
24	0.19	0.30	0.52	0.71	0.89

Table 5: Estimated power by simulation with $\alpha = 0.1$

Table 4 and 5 shows the estimated power for different sample sizes, effect sizes and significance levels. Corresponding entries to the real data set are in bold text (0.29 and 0.37). The results can be interpreted that with current estimated effect size, the probability of falsely accepting $\beta_2 = 0$ is high (over 0.5), even with the sample size doubled.

6 Conclusion and Discussion

6.1 Experiment Phase 1

- Phase I need multiple testing correction since analysis involves multiple simultaneous statistical tests.
- There is no significant statistical evidence to support that e-cigarettes and air have different impacts on critical Stem cells(LKS,LKS_SlAM) once multiple testing correction applied.
- The Statistical Power of Phase I was pretty low. There is only 60% statistical power with 6 mice in each group.
- Pilot study data indicates (fairly clearly) that just need higher n to reach desired significance level.

6.2 Experiment Phase 2

For the data analysis in phase 2, we take into account the weighted response (by number of cells) and correlation between observations over time, and thus used the generalized linear mixed-effect model to formulate them. Though the model point estimates do suggest that e-cig vapor exerts a downward selective pressure on HSC's, the final result shows no significant difference between Air and E-cig groups in terms of CD45.2 cell proportion.

For future investigation we propose the following perspectives for improvement:

- EDA suggests that the trend in the response over time may not be linear. Here, the non-linearity is probably slight enough to justify continuing to use a linear model, but future analyses observing a higher degree of non-linearity may find it useful to model the default group semi-parametrically using a generalized additive model (GAM), or perhaps to revise the scientific question such that a transformation of the response is motivated.
- The number of total cells at each blood draw and the time between blood draws vary, complicating the picture unnecessarily. Future experiments may benefit from more standardization regarding these operations.
- Similarly, knowing how many CD45.2 stem cells (particularly long-term stem cells) exist in both of the donor bone marrow mixtures would be helpful. Right now, we are treating the two donor

bone marrow mixtures as if they are comparable in every way except Phase 1 experimental group. However, experiment 1 results indicate that the stem cell donor bone marrow mixture likely contains fewer long-term stem cells than the air donor marrow, This would ostensibly set the treatment group stem cells at a disadvantage from the beginning.

- A more appropriate way to assess competitive advantage may be to measure the relationship between percentages and the *interaction* between treatment and time over the whole time period. This is especially true when, as mentioned above, the starting number of stem cells is unknown. In the current model, the parameter of interest is analogous to a difference in starting times - one car started earlier, so it is always ahead of the other. Adding an interaction with time into the model would allow for a difference in speeds - the car that starts later could catch and surpass the first one (which, incidentally, is what EDA suggests is occurring, though – contrary to current scientific knowledge – in favor of the e-cigarette-exposed stem cells)..

7 Listing of Tables and Figures

7.1 power graphs for other cell types, phase I experiment

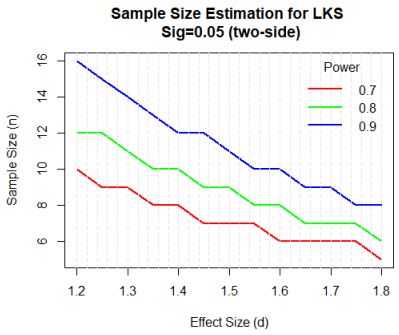

Figure 10: Est. Sample Size (sig=0.05)

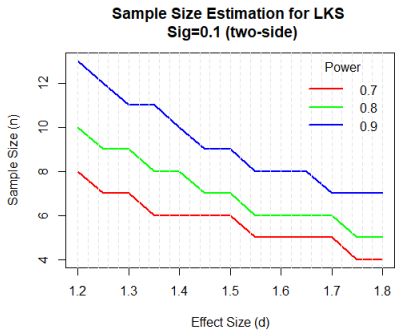

Figure 11: Est. Sample Size (sig=0.1)

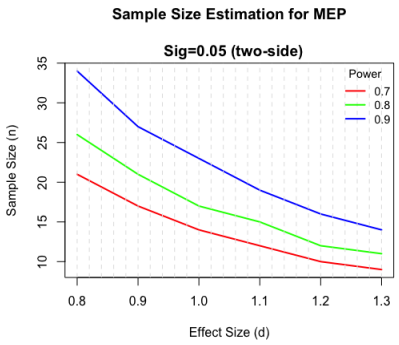

Figure 12: Est. Sample Size (sig=0.05)

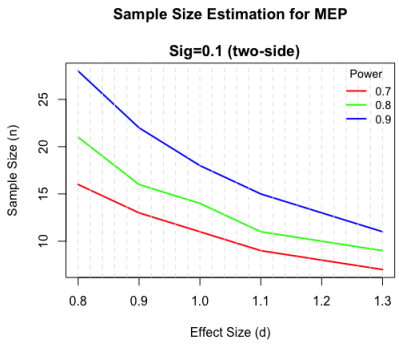

Figure 13: Est. Sample Size (sig=0.1)

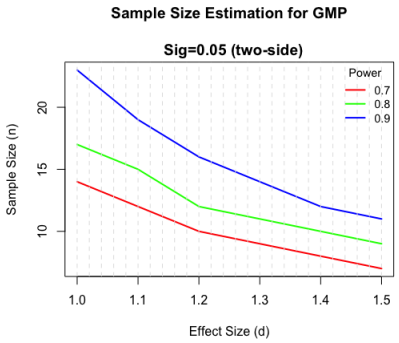

Figure 14: Est. Sample Size (sig=0.05)

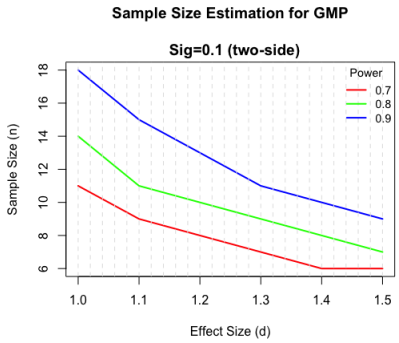

Figure 15: Est. Sample Size (sig=0.1)

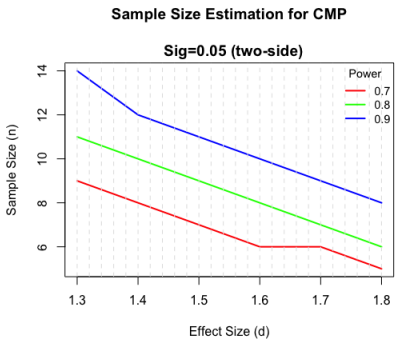

Figure 16: Est. Sample Size (sig=0.05)

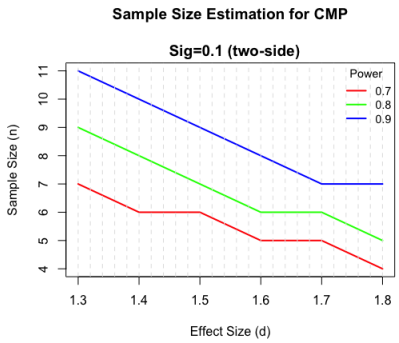

Figure 17: Est. Sample Size (sig=0.1)

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