Study Protocol and Statistical Analysis Plan

Body and Social Behavior

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INFLUENCE OF MILD INFLAMMATORY CHALLENGE ON CYTOKINES, AFFECT, AND SOCIALITY IN HUMANS; A RANDOMIZED CONTROLLED TRIAL USING THE INFLUENZA VACCINE.

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1. Description: This parallel-arm double-blind placebo-controlled randomized clinical trial examined the influence of a mild inflammatory challenge (the influenza vaccine) on various aspects of human affect, social perception, and sickness symptoms. *Please note, as this project was designed to address several scientific aims – we have chosen to submit an initial pre-registration of methods. Specific hypotheses and statistical tests will be outlined in amendments to this pre-registration.*

2. Design

Overview of Study design: Participants completed two in-person study sessions ~24 hours apart. All study visits were scheduled in the morning (7:00 AM – 11:00 AM). During the first session (1-hour), participants completed informed consent, a self-report baseline questionnaire, and a heartbeat counting task. They also provided a baseline blood sample, collected via venipuncture, and were administered an injection of either the active influenza vaccine or a sham vaccine (saline solution). During the second session (2-hours), participants again completed self-report questionnaires and had their blood taken via venipuncture. They then completed three tasks: a sociogram task, two social interaction tasks, and a bargaining game task. Participants were then debriefed and compensated $25/hour.

Study type: Experiment

Randomization & Blinding: Simple condition random assignment was implemented by UNC's Investigational Drug Services (IDS) pharmacy. Pre-filled (unlabeled) syringes were delivered by IDS to the University of North Carolina at Chapel Hill's Clinical and Translational research center (CTRC) in opaque stapled bags. These bags were opened by trained CTRC nursing staff—who dispensed the doses—immediately prior to drug injection. Primary research staff were not present for drug injection and participants were asked to look away during injection (and never saw the syringes) to maintain blinding.

3. Sampling Plan

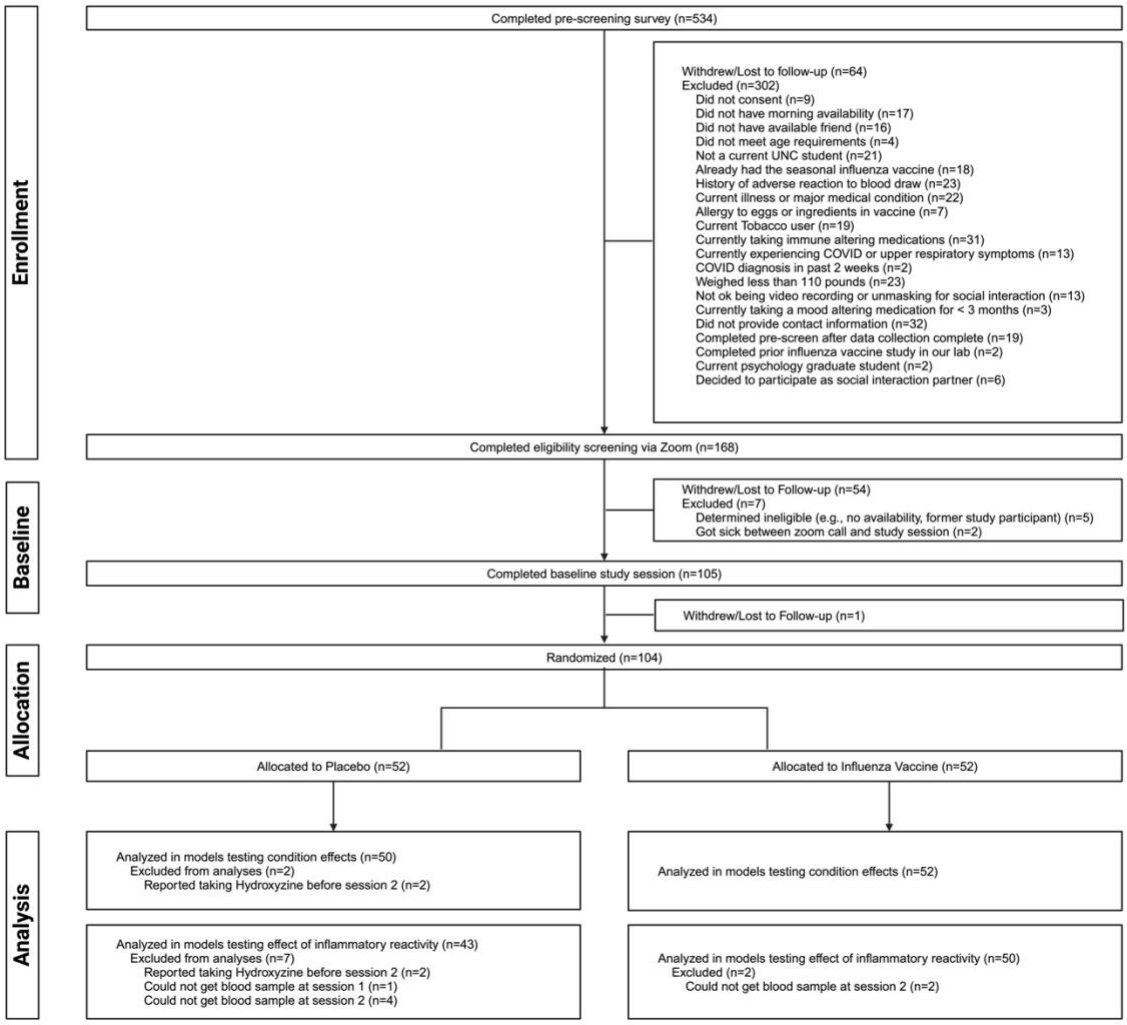
Existing data: As of the date of submission, the data exist, and we have accessed it.

Explanation of existing data:

- A combination of t-tests and chi-squared tests were conducted to assess for any failure in random assignment. Variables assessed included: Age, Assigned Sex at Birth (ASAB), Gender, Sexual Orientation, Race/Ethnicity, Annual Household Income, Subjective Socioeconomic Status, Depressive Symptoms, Perceived Stress, Sleep Health, menstrual status, use of birth control, waist-hip ratio, mental health diagnoses, use of mood-altering medications, baseline levels of circulating cytokines, and non-compliance with instructions to refrain from alcohol use taking OTC medications prior to study sessions.

- KM presented a poster at the annual PsychoNeuroImmunology Research Society conference (June 2024). For this poster, MF conducted basic t-tests to assess group differences in inflammatory cytokines, sickness behavior, social connection, state neediness, valence, and arousal at session 2.

Sample size:



Sample size rationale: A priori, we aimed to recruit 100 individuals (~50 females) ages 18-35. This sample size was selected based on a power-analysis conducted in G*Power (Faul et al 2007) indicating that a minimum of 90 participants (45 per condition) were necessary to achieve 80% power to detect a medium effect size of F^2=.15 for a within/between-subjects interaction (2 groups; 2 repeated measurements; 0.5 correlation between measures). We aimed to collect 100 instead of 90 in case of data loss due to, for example, participants not returning to the lab for their post-vaccine session.

Stopping rule: We concluded the study after reaching our target recruit goal (N=100), which happened to coincide with the start of the university's winter break, meaning clinics and services

needed to run sessions (IDS, CTRC) were closed. We also wanted to avoid the height of influenza season, which the CDC stated was January-February of that year.

4. Variables

Manipulated variables:

- **Inflammation.** All participants were assigned to receive either the active influenza vaccine (0.5 mL FluLaval Quadrivalent) or a sham vaccine (0.5 mL Sodium Chloride, i.e., saline) during session 1. At both sessions 1 and 2, approximately 6 mL of blood were collected via venipuncture. Final plasma samples were assayed for Interleuken-6 (IL-6), Interkeluken-10 (IL-10), Interferon-Gamma (IFN-γ), and Tumor Necrosis Factor-Alpha (TNF-α)) using a Simple Plex Cartridge Kit from R&D systems Inc. (Minneapolis, MN) containing IFN-gamma 3rd gen, IL-10 2nd gen, IL-10 2nd gen, IL-6 3rd gen, TNF-alpha 2nd gen cartridges for use with Human Plasma/Serum.

Measured variables/Indices:

Session 1

- **Self-report questionnaires:**
  - **Valence, Arousal, Dominance:** participants responded to prompts asking "how [pleasant or unpleasant/ activated or deactivated/ submissive (without control) or dominant (in control)] do you feel right now."
  - **Open ended feelings report:** Participants responded to the prompt, "Please write out in your own words how you feel right now. Please be as SPECIFIC and DESCRIPTIVE as possible about how you feel" using a free-text field.
  - **Sickness Symptoms** (Andreasson et al., 2018).
  - **Daily Health Behaviors:** Participants reported on exercise, sexual activity, and consumption of caffeine, cigarettes, other nicotine products, alcohol, and medications over the past 24-48 hours.
  - **Adapted Brief Pittsburgh Sleep Quality Index** (Sancho-Domingo et al., 2021; Gordon & Chen, 2016)
  - **RU-SATED Scale** (Buysse, 2014).
  - **Interoception Sensory Questionnaire** (Fiene et al., 2018)
  - **Two-way social support Scale** (Shakespeare-Finch & Obst, 2011)
  - **Physical Health Questionnaire-9** (Kroenke et al., 2001)
  - **The Perceived Stress Scale** (Cohen et al., 1983)
  - **Friend Questions:** Participants were asked to indicate how long they had known their same-gender friend (who was enrolled to participate as an interaction partner at session 2), how long they had been friends with their same-gender friend, their relationship satisfaction with their same-gender friend, an inclusion of other in the self-scale (Aron et al., 1992) measuring perceived self-other overlap between them and their same-gender friend, and the extent to which they could rely on and count on their friend.
  - **Couples Satisfaction Index (CSI) scales** (Funk & Rogge, 2007)
  - **Revised Cheek and Buss Shyness Scale** (Hopko et al., 2005; Cheek, 1983)
  - **The Psychoticism Scale** (Eysenck et al., 1985)
  - **Revised UCLA Loneliness Scale** (Russell et al., 1980)
  - **Self-Construal Scale** (Park & Kitayama, 2014)
  - **The Experiences in Close Relationship Scale** (Wei et al., 2007)
  - **Positivity Resonance Scale (3-item version)** (Major et al., 2018)

  - **Revised Self-Monitoring Scale** (Lennox & Wolfe, 1984)
  - **Approach and Avoidance Motivation Scale** (Elliot et al., 2006)
  - **Menstrual Status:** Participants indicated whether they menstruated, dates of their last and (anticipated) next period, cycle regularity, whether their cycle was predictable, and whether they took birth control. Menstrual day was calculated based on guidelines published in (Schmalenberger et al., 2021).
  - **Traditional Masculinity and Femininity Scale** (Kachel et al., 2016)
  - **Demographics:** Participants reported their age, sex, gender, race/ethnicity, sexual orientation, parental education, annual household income (for students, based on parents income), and subjective socioeconomic status and community status (Adler et al, 2000).
- **Heartbeat counting task** (Schandry, 1981): On each of 6 experimental trials, participants were asked to count their heartbeat for a variable period of time (25, 30, 35, 40, 45, and 50s in randomized order). After each trial, participants reported their subjective confidence in their count. Trials were interleaved with 30 second rest periods. This version of the Heartbeat counting task was implemented in Psychopy using scripts developed by (Legrand et al., 2022).

Session 2

- **Self-report questionnaires:**
  - **Valence, Arousal, Dominance:** (same as above)
  - **Open ended feelings report:** (same as above)
  - **Sickness Symptoms:** (same as above)
  - **Daily Health Behaviors:** (same as above)
  - **Adapted Brief Pittsburgh Sleep Quality Index:** (same as above)
  - **Social Connection:** Participants responded to 10-items assessing feelings of social disconnection (adapted from Moieni et al., 2015).
  - **Modified Positive and Negative Affect Schedule (PANAS):** Participants rated the extent to which they were experiencing each of 30 emotion words ranging in both their valence and arousal. Words were taken primarily from the expanded version of the PANAS (David & Clark, 2011) and the State Trait Anxiety Inventory (Spielberger et al., 2017).
  - **Physical Symptoms Endorsements:** Participants rated the extent to which they were experiencing each of 12 physical symptoms (fidgety, muscle aches/pains, poor appetite, sluggish, drained, fatigued, energized, wide awake, restless, powerful, heavy, and weak).
  - **State Connectedness and Neediness** (Rude & Burnham, 1995)
  - **Friend Questions:** Participants were asked to indicate relationship satisfaction with their same-gender friend (same as above) as well as how much they had been co-present with their same-gender friend in the last 24 hours, and how connected to their friend they felt over the past 24 hours.
- **Sociogram task.** The sociogram task followed the standard protocol of the task (Kitayama et al., 2009). In the task, participants were told they would have five minutes to draw a picture of their social network using circles to symbolize each individual in the network and lines to symbolize a relationship between two members of the network, starting by placing themselves in the picture. Participants were instructed to only include the first and last initial and the gender identity (either Male, Non-binary, or Female). Participants were shown an example of a hypothetical network and then asked if they had any questions before starting on their own. On a standard sheet of printer paper, participants drew their sociogram for five minutes before they were asked to stop by the research assistant.

- **Social Interaction task.**
    - **Fast-Friends Procedure:** The fast friends procedure followed standard protocol of this task (Aron et al., 1997; Sprecher, 2020), in which two people engaged in a structured conversation for 12-15 minutes in-lab. The conversation was structured around one of two sets of 14 "get acquainted" questions (see Aron et al., 1997), in which participants alternated asking questions from a set of question cards. After reading a question, the other person first answered and then the question-asker answered the same question. We had two sets of question cards that were matched on intimacy and depth and were counterbalanced between the primary participant's two interactions (with a close friend and stranger). The conversations were videorecorded, and physiology was assessed throughout.
    - **Pre-interaction survey:** Before the social interaction, participants independently and privately completed a set of items about anticipated feelings and expectations about the upcoming interaction. The set of items were drawn from existing work in relationship formation and relationship and affective science (e.g., IOS scale, see above; expected positivity resonance, adapted from Major et al., 2018). Social interaction partners were also asked about their prior night's sleep (see above) and demographics (see above).
    - **Post-interaction Survey:** Immediately following the interaction, both participants in the social interaction completed a post-interaction survey, independently and privately (in their own room). The set of post-interaction items were drawn from existing work on impression formation, relationship initiation, and relationship and affective science (e.g., meta-perceptions of liking, Boothby et al., 2018; perceived similarity and self-disclosure, Sprecher, 2021; feelings of social connection, Inagaki et al., 2020; perceived partner responsiveness, Reis et al., 2017). Social interaction partners of the primary participant were also asked whether they thought the primary participant had received the influenza vaccine.
- **Bargaining game task.** Participants completed a two-part decision-making task adapted from standard protocols of behavioral economics games, including the Ultimatum Game and the Dictator Game (List, 2007; Nowak et al., 2000). In both parts of the task, participants played a bargaining game in which they were asked to make decisions about how to split different stakes of money between themselves and another person. On each of 90 experimental trials in the first part of the task, participants were asked to either "accept" or "reject" a money offer made by one of 90 different proposers on a stake of either $9, $10, or $11. The proposed offers ranged from 10-50% of the stake. Participants were told that if they accepted an offer, then both them and the proposer would get the proposed split of money, and that if they rejected an offer, then neither them nor the proposer would get any money. In the second part of this task, participants took on the proposer role and were instructed to indicate how much of different money stakes they would like to offer to future responders in the study.
    - **Post-task survey:** Immediately following the task, participants reported their subjective ratings of five different offers they responded to during part one of the task. Specifically, for each of the five offers, participants were asked to write a couple of sentences answering what they thought about the offer and how the offer made them feel.
- **Debriefing.** Primary participants were debriefed at the end of the study, beginning with answering a few questions such as, 1) which vaccine condition do you think you were in?, 2) how confident are you that you were in that condition?, and 3) why do you think you

were in that condition? Then, participants were offered an opportunity to view and consent to additional uses of their video data. They were informed of their vaccine condition assignment at the end of the study and if they were in the placebo condition, given the opportunity to receive the influenza vaccine.

5. Analysis

Transformations: Cytokine measurements that were below the limit of detection for assay were replaced with the lower limit of detection value. Cytokine measurements that were detectable, but more than 3 standard deviations above the mean were winsorized and replaced with that value (i.e., *Mean* + 3 SDs). Then, values for IL-6, IL-10, IFN-γ, and TNF- α were log-10 transformed. Inflammatory reactivity to the vaccine was computed as the change in log-transformed concentrations of circulating cytokines from pre- to post-vaccine.

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AMENDMENT 1: Testing for basic differences in physical symptoms, affectivity, and social connection.

1. Variables used

- Demographic data
- S2 daily health behaviors
- Circulating concentrations of inflammation at session 1 and session 2
- Sickness Symptom Questionnaire (SicknessQ)
- Physical Symptom Endorsements for "Fatigue" and "Muscle aches/pains"
- Modified Positive and Negative Affect Schedule (PANAS)
- Adapted 5-item brief Pittsburgh Sleep Quality Index
- 10-items assessing social disconnection

2. Analysis Plan

Statistical models: Statistical models will assess 5 broad scientific aims.

Scientific Aim 1: Inflammatory Cytokines. To test whether treatment groups differed in circulating levels of pro-inflammatory cytokines following vaccine administration, we will run repeated-measures analyses of variance (RM-ANOVA; Winkens et al 2007). Inflammatory cytokines at both sessions will be treated as the criterion, session number (i.e., time) will be treated as a within-subjects predictor, and condition assignment (placebo vs. influenza vaccine) will be treated as a between-subjects predictor. RM-ANOVA models will be run separately for each of the four inflammatory cytokines assayed. Significance will be assessed at $p<0.05$. We hypothesize a significant interaction between condition and time on inflammation such that participants in the influenza condition will have higher levels of all four markers of inflammation than participants in the placebo condition at session 2 but not at session 1.

Scientific Aim 2: Sickness Symptoms. To test whether treatment groups differed in sickness symptoms following drug administration, we will complete a series of analytic steps:

1. Given that the Sickness Symptom Questionnaire (SicknessQ) has primarily been validated in samples treated with endotoxin (a potent inflammatory challenge), we will begin by conducting confirmatory factor analysis with 1 factor on scale items. Good model fit for CFA will be represented by RMSEA <=.08, CFI >= .95, and TLI >= .90 (Hu & Bentler, 1999; Schreiber, Nora, Stage, Barlow, & King, 2006). We will then run t-tests to assess for group differences in overall sickness symptom scores. Significance for t-tests will be assessed at $p<0.05$.
2. We will also run separate two-tailed independent-samples t-tests to assess for group differences in individual sickness symptoms (i.e., each item of the SicknessQ). Significance will be assessed at $p<0.05$.
3. Finally, we will run two additional two-tailed independent-samples t-tests to assess for group differences in endorsements of two physical symptoms not included in the SicknessQ but commonly assessed when using this and similar methods (Eisenberger et al., 2009; Kuhlman et al., 2018): "*fatigue*" and "*muscle aches/pain*." Significance will be assessed at $p<0.05$. Significance will be assessed at $p<0.05$.

Scientific Aim 3. Affect. To test whether treatment groups differed in felt affect following vaccine administration, we will run 4 two-tailed independent-samples t-tests to assess for group differences in positive high arousal (PH) affect, positive low arousal (PL) affect, negative high

(NH) arousal affect, and negative low (NL) arousal affect respectively. PH, PL, NH, and NL will be computed using the Positive and Negative Affect Schedule (PANAS) word endorsements. Specifically, PH will be operationalized as the mean endorsement across items: *amused, determined, enthusiastic, excited, happy, inspired, proud, strong, active, and alert*. PN will be operationalized as the mean endorsement across items: *Angry, Irritable, Nervous, Scared, Upset, Afraid, Ashamed, Distressed, Frustrated, Hostile, and Jittery*. NL will be operationalized as mean endorsement across items: *bored, guilty, sad, and worn out.* PL will be operationalized as mean endorsement across items: *attentive, calm, interested, grateful, and relieved*. Significance for t-tests will be assessed at $p<0.05$.

Scientific aim 4. Sleep. We will run three two-tailed independent-samples t-tests to assess for group differences in the subsequent night's sleep following vaccine-administration. First, we will assess for group differences in that night's sleep as operationalized using an index of items adapted from the Brief Pittsburgh Sleep Quality Index. These five items assess five facets of sleep—quality, quantity, daytime dysfunction, latency, and nighttime awakenings—and will be combined into a "Sleep Quality Index." Next, we will run two additional models testing for group differences in two facets, subjective sleep quality (1-item) and subjective quantity (1-item), separately. Significance for all t-tests will be assessed at $p<0.05$.

Scientific Aim 5. Social Connection. We will run a two-tailed t-test to assess for group differences in felt social connection. Social connection will be operationalized as averaged scores on 10-items assessing feelings of social disconnection. Significance for t-tests will be assessed at $p<0.05$.

Scientific Aim 6: Models using continuous measures of circulating cytokines. To assess evidence for dose-dependency effects between circulating cytokines and outcomes-of-interest (same outcomes described in scientific aims 2-5), we will conduct a series of linear regression models using data from only the subset of participants assigned to the influenza vaccine condition (n=52). Models will be run separately for each outcome-of-interest and for each cytokine and will take the general form:

1. $outcome_i = \beta_0 + \beta_1(\Delta\, inflammation) + \beta_2(BL\, inflammation)$
2. $outcome_i = \beta_0 + \beta_1(\Delta\, inflammation) + \beta_2(BL\, inflammation) + \beta_3(ASAB) + \beta_4\left(\frac{waist}{hip}\right)$

**Note that we will only control for baseline inflammation in models if baseline inflammation is significantly correlated with change in inflammation (*Dalecki & Willits, 1991; Llabre et al., 1991; O'Connell et al., 2017*).*

Sensitivity analyses will also be run to assess whether results are robust when individuals are excluded who reported a diagnosis of depression (n=4), took an SSRI the day of the session (n=4), or who were non-compliant prior to study session 2 (3 participants drank alcohol and 5 participants took pain-killers). Sensitivity analyses will be run excluding groups one by one to maximize power.

Power:

Sensitivity Analysis for RM-ANOVA: See "sample size rational" in main study pre-registration. Sensitivity analyses conducted in g*power (Faul et al 2007) for ANOVA (repeated measures, within-between interaction) with α=0.05, (1- β)=0.80, number of n_{groups}=2, $n_{measurements}$=2,

$n_{participants}$=102, r=0.5, suggested that we would be adequately powered to observe a medium effect size f^2=0.14.

Sensitivity Analysis for t-tests: Sensitivity analysis conducted in g*power for a two-tailed t-test (independent means) with α=0.05, (1- β)=0.80, n_1=50, n_2=52 suggested that we would be adequately powered to observe powered to observe a medium effect size d=0.56.

Sensitivity Analysis for linear regression: Sensitivity analysis for simple regression (fixed model, single regression coefficient) for a two-tailed t-test with α=0.05, (1- β)=0.80, $n_{participants}$=52, and $n_{predictors}$=4 suggested that we would be adequately powered to observe a medium effect size f^2=0.16.

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