1. INTRODUCTION

One of the first senses to develop, touch is a unique form of nonverbal communication and plays an essential role in shaping human interactions and mood [1, 2]. The right kind of touch-sensations can stimulate our bodies and produce physical, emotional, and cognitive improvements by moderating the interplay between psychological factors and neural, endocrine and immune systems [3, 4]. Accordingly, tactile massage with long, calm stroking movements (Touch Massage or TM) is often used to decrease stress, anxiety and pain and engender a sense of wellbeing and healthiness [2, 5-7]. As our understanding of the health effects of touch grows, so too do the opportunities to leverage touch-sensation as a therapeutic adjuvant. However, efficacy studies investigating the linkages between touch sensation, immune mediators and emotional states could be useful for guiding practitioners delivering touch therapy.

Keywords: Touch-sensation, Salivary cytokine, Emotion
collections in real-life environs [18, 19]. Emerging technologies now enable the measurement of cytokines expressed in saliva and open up the possibility of studying inflammatory processes in saliva in response to stressors [20].

Of the 300+ cytokines identified to date, several including IL-1β, tumor necrosis factor alpha (TNF-α), and IL-6 expressing in saliva have been associated with stress and emotional states [21]. Identifying a selected panel of salivary cytokines reflective of stress status and mood would help advance temporal explorations of the interactions between touch and emotional regulation. We postulated that combining inflammatory cytokines, implicated in a broad variety of stress-related diseases, would lead to more objective ways to evaluate the effects of TM interventions on happiness and arousal levels. The aim of our study was to evaluate how the levels of selected salivary cytokines varied in response to a TM intervention, and to construct a cytokine panel that best reflects happiness and arousal levels related to TM.

2. MATERIALS AND METHODS

2.1 Participants

Study participants were 40 healthy women aged 33 – 37 years (34.7 ± 1.4 yrs.) and recruited from the Tokyo Metropolitan area using a combination of e-mails, posters, and flyers. All subjects were single, employed and had an annual income more than 3 million JPY (≈ $ 27,000). Study exclusion criteria were poor oral hygiene, smoking, drug and/or alcohol abuse, pregnancy and/or nursing, acute and/or chronic infections, psychiatric diseases, and any sort of medication. All participants signed informed consent after arrival at the laboratory and were reimbursed for their participation. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee of Shinshu University. In addition, we recruited seven women as the interventionists aged 30 – 39 years (36.0 ± 3.1 yrs.) for delivering a standardized TM intervention.

2.2 Design

We employed a crossover design, with the TM intervention as the within-subjects variable. Sample size was calculated based on earlier research that found improved mood (calmness, awakeness) following a TM [8, 22]. To obtain a medium effect size (0.25) and acceptable power (i.e. 0.8; with alpha set at 0.05, one-tailed), the calculated sample size required was 40 subjects.

2.3 Procedure

Participants were tested individually in a quiet room with controlled temperature (25°C) and humidity (40%) and devoid of any visual stimuli. All tests were conducted within the same time window (10:00 a.m. – 2:00 p.m.). The seven TM interventionists were trained and calibrated in the delivery of a manualized upper-back massage in order to create a reproducible, structured protocol for delivering a distinct dose of the TM intervention [22, 23]. The TM protocol specified the body region (upper back), the time allocated (15-minutes), the strokes to be use and the sequence.

The study was carried out in two similar sessions conducted on two consecutive days. Participants were required to refrain from drinking liquids or chewing gum for at least 30 minutes before each session. At the baseline session, the consented participants completed emotional indicator questionnaires described below. Then, TM recipients were randomly assigned to receive the either the TM intervention or control condition in order to control for any order effect. Those in the control condition, underwent the same series of questionnaires and saliva sampling, but received no TM intervention.

Figure 1 summarizes the experimental protocol. Each recipient was seated in the test room, allowed to acclimatize for 5 minutes and the questionnaires administered. An initial saliva sample (S₀) was gathered with a collection tube (MS-50, Japan Medical Ltd., Tokyo, Japan) using the passive-drool method. Approximately 150 μL of saliva was collected over a 1 minute period at each collection. To rule out chronic periodontitis that would influence the concentrations of inflammatory and anti-inflammatory cytokines [24], a Perioscreen® (Sunstar Inc., Osaka, Japan) strip was placed into the saliva tube and the color change was noted. Each saliva tube was immediately stored on ice and subsequently frozen at –80°C until analysis.

![Figure 1: Experimental Protocol](image)

BL: Baseline acclimatization
TM: Touch Massage intervention
S₀-S₆₀: Saliva collection at 20 minute intervals
Q₀-Q₆₀: Questionnaire administration at 20 minute intervals
In those participants assigned to the TM intervention, the TM interventionist conducted a 15-minute massage of the recipient’s upper back using slow strokes with gentle pressure (Figure 2) [23]. Subjective evaluations \( (Q_{0-60}) \) and saliva sampling \( (S_{0-60}) \) were repeated at 20-min intervals.

### 2.4 Subjective Measures

Subjective experience of the recipient’s happiness and arousal levels in both the intervention and control sessions was captured as follows.

a) Mood was measured with the UWIST Mood Adjective Check List [25, 26], using a validated Japanese adaptation [27]. We used subscales to capture the dimensions of state mood: Energetic Arousal (EA) and Tense Arousal (TA). Respondents rated the degree to which each of the adjectives described their current mood on a four-point Likert-type scale. The convergent validity of the Japanese version is similar to the original, English-language. As summarized by Figure 1, the checklist was administered four times in each session. TA scores (ranging from feeling calm to feeling nervous) and EA scores (ranging from feeling sleepy to feeling awake) were calculated from the 20 adjectives [28].

b) A self-assessed questionnaire of psychological state of happiness and arousal levels (happiness and arousal questionnaire) was developed, consisting of two verbal descriptors: happiness and arousal. The intensity of the emotions was captured on a five-point scale: strongly disagree (1), somewhat disagree (2), neutral (3), somewhat agree (4) or strongly agree (5). Each happiness and arousal questionnaire was administered four times (Figure 1; \( Q_{0-60} \)) and results were compared between the TM and control conditions.

c) A self-assessed questionnaire of touch-sensation (touch questionnaire) was developed, consisting of two adjectives: comfortable with touch-sensation and uncomfortable on being touched by another person. The intensity of the emotions was captured by a two-point scale: agree (1), or disagree (2). The questionnaire was administered at the end of each session.

### 2.5 Salivary Cytokine Assays

Collected saliva samples were de-identified and stored at −80°C and, just prior to analysis, thawed to 4°C in a refrigerator. Finally, the saliva samples were brought to room temperature (24°C). Next, the saliva samples were centrifuged at 1,500 × g for 15 min. A micropipette was then used to sample a fixed aliquot of each sample (50 μL) for subsequent analysis.

Cytokine levels in saliva were measured using a validated multiplex bead array assay (Bio-Plex) according to the manufacturer’s instructions [29-32]. The targets included twenty seven cytokines (IL-1ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, C-C motif chemokine ligand 11 (CCL11, Eotaxin), fibroblast growth factor 2 (FGF-2), colony stimulating factors 2 (CSF2, GM-CSF), colony stimulating factors 3 (CSF3, G-CSF), interferon gamma (IFN-γ), TNF-α, C-X-C motif chemokine ligand 10 (CXCL10, IP-10), C-C motif chemokine ligand 2 (CCL2, MCP-1), C-C motif chemokine ligand 3 (CCL3, MIP-1α), C-C motif chemokine ligand 4 (CCL4, MIP-1β), platelet-derived growth factor-BB (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES), and vascular endothelial growth factor (VEGF). All samples were analyzed at the same day to avoid inter-assay variation. The Bio-Plex immunoassay system used human cytokine panels, and the plates were

![Figure 2: Structured, reproducible protocol for delivering distinct dose of TM intervention to a specified region](image-url)
read on a Bio-Plex Array Reader (Bio-Plex 200 System and Bio-Plex Manager Version 6.1, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The results from the de-identified samples were entered into a structured database by research staff not directly involved in subject treatment or sample assays.

2.6 Statistical Analysis and Construction of Prediction Model

The statistical analyzes were performed with the Statistical Package for Social Sciences (SPSS) version 25 (Advanced Analytics, Inc. Tokyo, Japan). Unless otherwise stated, continuous data are summarized as mean ± standard deviation (SD). A value of p < 0.05 was considered significant. Statistical analysis was not performed if the number of data points missing from one group exceeded half of all data points.

A prediction model was constructed using following procedures.

Step I: Prior to prediction model construction, each salivary cytokine level was verified by calculating its skewness and kurtosis from a histogram. The salivary cytokine levels were subjected to log-transformation and standardizing, and those below the limit of detection were given a value of half the detection limit [33, 34].

Step II: Wilcoxon signed rank tests were performed for salivary cytokine levels between 0 min ($S_{0}$) and other time-periods ($S_{20-60}$, Figure 1).

Step III: A correlation analysis was performed for all of the pairwise salivary cytokines to avoid collinearity. We excluded salivary cytokines that had correlation coefficients > 0.7 with the selected cytokine.

Step IV: A multiple conditional logistic regression analysis (Bell Curve for Excel, Social Survey Research Information Co., Ltd., Tokyo, Japan) was conducted to examine the association between salivary cytokines and the presence of touch-sensation. Two combination of time-periods were examined i) 0 min ($Q_{0}$) and 20 min ($Q_{20}$), Case 0-20 min; and ii) 0 min ($Q_{0}$) and 60 min ($Q_{60}$), Case 0-60 min. Odds ratios (ORs) and their 95% confidence intervals (95% CIs) for the presence of touch-sensation were estimated for each salivary cytokine. To construct a prediction model, candidate salivary cytokines were selected according to the following procedures. First, we nominated as the initial candidate the salivary cytokine with the smallest p-value in the aforementioned multiple conditional logistic regression analysis. Then, we identified another candidate salivary cytokine with the next smallest p-value and eliminated some of the remaining salivary cytokines in the case that had correlation coefficients with the second selected salivary cytokine > 0.7. These procedures were repeated until candidate salivary cytokines were finalized. By applying a backward elimination method to a logistic regression model with all possible candidates of salivary cytokines, we constructed a prediction model for the presence of touch-sensation [35].

Step V: Model performance was assessed with a discrimination test using receiver operating characteristic (ROC) curve analysis [36]. ROC curves were generated to investigate the discriminatory power of the salivary cytokine levels. The areas under the curves (AUCs) were calculated to provide an overall summary of the detection accuracy of the salivary cytokine levels, and were empirically classified into three levels: poor when $0.50 \leq \text{AUC} < 0.69$, good when $0.70 \leq \text{AUC} < 0.89$, and excellent when $0.90 \leq \text{AUC} < 1$.

3. RESULTS

3.1 Subjective Evaluations

On the UWIST mood adjective checklist, the Touch Arousal scores at 20, 40, and 60 min were significantly different between the TM intervention and control sessions (p < 0.05). In contrast, the Energetic Arousal scores showed no significant differences between the two groups.

Figure 3 shows the results of the happiness and arousal questionnaire comparing the two groups. The happiness level scores at 20, 40, and 60 min were significantly different between the groups (p < 0.05) as were the between-group arousal level scores at 40 and 60 min (p < 0.05).

On the touch questionnaire, 34 participants reported that the touch-sensation felt comfortable whereas 6 participants were uncomfortable. Additionally, two participants answered that they do not enjoy being touched by another person, whereas 38 participants did not express any distaste.

3.2 Multiplex Analysis of Salivary Cytokines

In each group, one set of saliva samples tested positive for occult blood and the two samples were excluded from salivary cytokine analysis. Among the 27 target cytokines, only 21 could be analyzed due to low sensitivity of the Bio-Plex system to the remaining 6 (IL-4, IL-5, IL-13, IL-15, GM-CSF, and PDGF-BB, Figure 4). The salivary cytokine levels in the control group ranged between 0.01 (IL-10, IL-12p70, and Eotaxin) and 19694.20 (IL-1ra) pg/mL. In contrast, salivary cytokine levels with TM intervention
ranged between 0.01 (IL-10 and IL-12p70) and 16979.70 (IL-1ra) pg/mL. A five-digit difference was observed in the absolute values of salivary cytokine levels.

### 3.3 Statistical Analysis and Prediction Model Construction

Step II: For Case 0-20 min (between 0 and 20 min), 13 salivary cytokines (IL-6, IL-7, IL-9, IL-10, IL-12p70A, IL-17A, Eotaxin, TNF-α, MCP-1, MIP-1α, MIP-1β, RANTES, and VEGF) satisfied following both conditions in that significant differences were observed with TM intervention (p<0.05) and no significant differences were observed without TM intervention (Table 1). For Case 0-60 min (between 0 and 60 min), we identified 11 salivary cytokines (IL-2, IL-7, IL-8, IL-9, IL-10, IL-17A, Eotaxin, FGF-2, MIP-1α, MIP-1β, and VEGF) that satisfied following both conditions (Table 1).
Step III: For Case 0-20 min, 9 salivary cytokines were nominated as candidates for a prediction model after excluding those correlation coefficients exceeded 0.7: IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-17A, MCP-1, MIP-1α, and VEGF. Using the same criterion for 0-60 min, 7 salivary cytokines were nominated as candidates: IL-7, IL-8, IL-9, IL-10, IL-17A, FGF-2, and MIP-1β.

Step IV: As seen in Table 2, IL-7 showed the smallest p-values in the Case 0-20 min model and was selected as the initial candidate. Also, IL-9 showed the smallest p-values in the Case 0-60 min model and was selected as the initial candidate. The backward elimination method revealed two multivariable prediction models (logistic models) for the presence of TM intervention as follows (p < 0.05, Table 3):

\[
\text{Case 0-20 min model:} \quad z = 4.572 \text{ IL-17A} \\
\text{Case 0-60 min model:} \quad z = 1.580 \text{ IL-9} + 1.960 \text{ IL-17A}
\]

Step V: We performed ROC analysis to evaluate the above two prediction models for the presence of touch-sensation (Figure 5). In the Case 0-20 min model, the AUC, sensitivity, and specificity of the optimal cutoff points were 0.955, 0.923, and 0.923, respectively. In the Case 0-60 min model, the AUC, sensitivity, and specificity of the optimal cutoff points were 0.916, 0.872, and 0.846, respectively, while the corresponding values in the IL-9 model were 0.865, 0.872, and 0.821, and those in the IL-17A model were 0.896, 0.769, and 0.897, respectively. Single-cytokine models presented lower AUCs (0.865 – 0.896) than the multi-cytokine IL-9 and IL-17A models (0.916). Both logistic models thus demonstrate “excellent” capability for discriminating between two time-periods.

### Table 1: Wilcoxon signed rank tests comparing both time-periods with touch-sensation data

| Cytokine | Case 0-20 min | Case 0-60 min |
|----------|---------------|---------------|
|          | p-value       | p-value       |
| IL-17A   | 0.0000008     | IL-17A        | 0.00002       |
| IL-7     | 0.00001       | FGF-2         | 0.0001        |
| Eotaxin  | 0.00002       | MIP-1β        | 0.0003        |
| MIP-1β   | 0.00009       | IL-7          | 0.001         |
| TNF-α    | 0.0002        | IL-2          | 0.001         |
| RANTES   | 0.0003        | MIP-1α        | 0.001         |
| MIP-1α   | 0.001         | IL-9          | 0.002         |
| IL-9     | 0.002         | IL-8          | 0.003         |
| IL-6     | 0.008         | IL-10         | 0.005         |
| IL-10    | 0.013         | Eotaxin       | 0.005         |
| VEGF     | 0.016         | VEGF          | 0.016         |
| IL-12p70 | 0.035         |               |               |
| MCP-1    | 0.046         |               |               |

### Table 2: Logistic regression analysis

#### Case 0-20 min

| Cytokine | Regression coefficient | Standard error | p-value | Odds ratio (OR) | 95% confidence interval (95% CI) |
|----------|------------------------|----------------|---------|----------------|----------------------------------|
| IL-6     | 1.525                  | 0.645          | 0.018   | 4.595          | 1.298 – 16.259                   |
| IL-7     | 2.831                  | 0.882          | 0.001   | 16.954         | 3.007 – 95.581                   |
| IL-9     | 1.587                  | 0.615          | 0.010   | 4.889          | 1.464 – 16.325                   |
| IL-10    | 1.884                  | 0.678          | 0.006   | 6.577          | 1.740 – 24.858                   |
| IL-12p70 | 0.983                  | 0.537          | 0.008   | 2.671          | 0.932 – 7.658                    |
| IL-17A   | 4.572                  | 1.493          | 0.002   | 96.780         | 5.184 – 1806.721                |
| MCP-1    | 0.867                  | 0.535          | 0.015   | 2.380          | 0.834 – 6.791                    |
| MIP-1α   | 1.641                  | 0.579          | 0.005   | 5.158          | 1.658 – 16.046                   |
| VEGF     | 1.447                  | 0.736          | 0.049   | 4.250          | 1.004 – 17.995                   |

#### Case 0-60 min

| Cytokine | Regression coefficient | Standard error | p-value | Odds ratio (OR) | 95% confidence interval (95% CI) |
|----------|------------------------|----------------|---------|----------------|----------------------------------|
| IL-7     | 1.718                  | 0.606          | 0.005   | 5.572          | 1.700 – 18.266                   |
| IL-8     | 1.065                  | 0.404          | 0.008   | 2.902          | 1.316 – 6.400                    |
| IL-9     | 2.463                  | 0.816          | 0.003   | 11.740         | 2.371 – 58.140                   |
| IL-10    | 2.003                  | 0.767          | 0.009   | 7.414          | 1.649 – 33.340                   |
| IL-17A   | 2.216                  | 0.766          | 0.004   | 9.168          | 2.042 – 41.165                   |
| FGF-2    | 0.321                  | 0.418          | 0.442   | 1.379          | 0.608 – 3.126                    |
| MIP-1β   | 1.320                  | 0.463          | 0.004   | 3.742          | 1.509 – 9.274                    |
4. DISCUSSION

Our study aimed to identify and construct a panel of salivary cytokines that could quantify and predict happiness and arousal levels related to TM. A series of questionnaires, including the UWIST mood adjective checklist, provided a qualitative evaluation of the happiness and arousal levels following a standardized TM intervention. A multiplexed immunoassay determined the levels of 27 select cytokines in saliva samples collected at corresponding time points. We postulated that a TM would lead to an increase in emotional indicators of well-being (happiness and arousal) and that these mood changes would be captured by the variation in select salivary cytokines.

Consistent with our postulate, the results revealed temporal changes in happiness and arousal scores, captured by the UWIST checklist, following the TM intervention. Although similar at baseline, the differences in the mood dimensions of tense arousal (TA) significantly differed between the experimental and control conditions as the TM session progressed. Furthermore, the changes in the mood indicators were in the expected direction; that is, following a TM, the recipients reported increased feelings of calmness and energy, as compared to baseline. Conversely, in the control session, the mood changes did not differ significantly as compared to baseline.

The levels of salivary cytokines varied according to the happiness and arousal emotions associated with the TM. Several cytokines differed significantly between the intervention and control conditions and a panel constructed from select salivary cytokines showed particular promise in discriminating the positive emotions were evoked. The variations in the levels of salivary cytokines appeared to index the mood states of the participants. In particular, the levels of IL-7, IL-8, IL-9, IL-10, IL-17A, FGF-2, and MIP-1β differed between the intervention and control conditions. A combination of select salivary cytokines showed particular promise in measuring the mood states. Specifically, ROC analysis showed that the IL-9 and IL-17A models had “excellent” capability for discriminating changes in mood states after a TM. Additionally, the multi-cytokine IL-9 and IL-17A models showed higher performance for discriminating the effects of TM than any individual cytokine.

Cytokine profiles of depression and anxiety symptoms have been attracting a great deal of attention. Stress-related psychophysical outcomes could manifest emotions [37]. IL-9 is considered a pleiotropic cytokine, and elevated IL-9 has been linked with increased risk for allergenic lung inflammation [38, 39]. Karlsson and colleagues reported that maternal depression and anxiety symptom

| Case       | Cytokine | Regression coefficient | Standard error | p-value | Odds ratio (OR) | 95% confidence interval (95% CI) |
|------------|----------|------------------------|----------------|---------|----------------|---------------------------------|
| 0-20 min   | IL-17A   | 4.572                  | 1.493          | 0.002   | 96.780         | 5.184 - 1806.721                |
| 0-60 min   | IL-9     | 1.580                  | 0.736          | 0.032   | 4.856          | 1.148 - 20.535                 |
| 0-60 min   | IL-17A   | 1.961                  | 0.871          | 0.024   | 7.103          | 1.288 - 39.171                 |

Figure 5: ROC curve analysis of TM and emotions. A: Case 0 – 20 min, and B: Case 0 – 60 min
scores in mid-pregnancy were associated with increased serum levels of Th2-related cytokines such as IL-9, but no earlier studies assessed IL-9 in the context of prenatal depressive or anxiety symptoms [40]. Li et al. mentioned that paoniflorin pretreatment reversed depressive-like behaviors and serum inflammatory cytokine levels including IL-9 [41].

Stress has been shown to suppress immune function and increase susceptibility to inflammatory disease and psychiatric diseases. CD4(+) CD25(+) regulatory T (Treg) cells are prominent in immune regulation. IL-17A (or IL-17) is a potent pro-inflammatory cytokine produced by activated T lymphocytes [42]. Pallavi and colleagues reported that anxiety scores negatively correlated with IL-17 [43]. Girardi et al. suggested that work-related stress may be associated with inflammation biomarkers such as IL-17A, and negative affectivity may influence the stress process affecting the exposure to psychosocial stressors [44].

Collectively, our results complement and extend previous research on using quantitative methods to clarify the relationship between touch-sensation and emotion regulation.

5. CONCLUSION

TM enhances mood states in healthy individuals and these are manifest in the varying levels of select salivary cytokines. These variations may be leveraged to measure the effects of touch-therapy on mood. In particular, a panel of salivary cytokine demonstrated “excellent” capability for discriminating changes in happiness and arousal levels after touch-therapy. Leveraging peripherally-expressed cytokines as representations of the biological stress responses provides opportunities to explore and quantify the relationships between touch sensation, immune mediators and emotional states.

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CONFLICT OF INTEREST STATEMENT

The authors state that they have no conflict of interests.

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AUTHOR CONTRIBUTIONS

M. Yamaguchi designed, performed, and analyzed the experiments and wrote the manuscript as the corresponding author. T. Sekine contributed to the data curation. V. Shetty supervised the research work and contributed to the manuscript editing. All authors contributed to the discussion of the results presented.

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