The effect of expressive writing on wound healing: Immunohistochemistry analysis of skin tissue two weeks after punch biopsy wounding

Running title: Effect of expressive writing on wound healing

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Abstract

Objective : To investigate the effects of expressive writing and its timing (pre or post wounding) on re-epithelialisation and leucocyte subsets within healing tissue. We previously showed expressive writing pre-wounding improved re-epithelialisation. Here we investigate cellular processes in the wound.

Methods: In a 2(writing content) x 2(writing timing) randomised trial, 122 participants were randomised to perform either expressive or control writing, before or after a 4mm punch biopsy wound. On day 14 post-wounding, participants had a 5mm punch biopsy of the initial wound. Seven of 16 primary registered outcomes were analysed, including re-epithelialisation from two photographs of the 4mm biopsy (previously reported). This paper reports immunohistochemistry analysis of five primary outcomes - Langerhans cells, immune cell activation (HLA and CD3+), and macrophages (CD68 and MPO) - in the 5mm biopsies in a random sample of 96 participants.

Results: Participants who performed either writing task pre-wounding had greater Langerhans cell infiltration, than those who wrote post-wounding ($F_{(1,85)}$=7.86, p=.006, η_p^2=.08). Those who performed expressive writing also had greater Langerhans cell infiltration than those who performed control writing ($F_{(1,85)}$=4.00, p=.049, η_p^2=.04). There were no significant group or interaction effects on immune cell activation or macrophages. Healed wounds on day 10 had lower levels of macrophages (z = -1.96, p = .050), and CD3+ cells (z = -1.99, p = .046) than non-healed wounds.

Conclusion : Langerhans cells in the healing skin are affected by the timing and topic of writing. More research is needed to further explore timing and corroborate these results.

Clinical Trials Registration: Registered at https://www.anzctr.org.au/ (Trial ID: ACTRN12614000971639)

Keywords: expressive writing, histology, punch biopsy, wound healing,

1. Introduction

Expressive writing is an intervention whereby people typically write for 20 minutes a day for three consecutive days about their deepest thoughts and emotions regarding a previous traumatic event or stressor [1]. Expressive writing helps people to create a narrative structure for the past event, stimulating cognitive processing and reducing negative affect when later recalling the event [1]. Expressive writing has been found to be beneficial not only in terms of self-reported health, psychological well-being and general functioning [2, 3], but also for immunological function. For example, compared to a control writing task, expressive writing has been shown to result in significantly higher antibody levels six months after a hepatitis B vaccine [4], greater lymphocyte proliferation after mitogen stimulation [5], and increased CD4+ lymphocyte counts and lower viral loads in HIV patients [6].

Expressive writing performed prior to an experimental punch biopsy wound has also been found to improve wound healing rates, compared to control writing [7-9]. For example, Weinman and colleagues [9] found that participants who performed expressive writing prior to receiving a 4mm punch biopsy had significantly smaller wounds after 14 days, as measured by a high-resolution ultrasound, than whose who performed control writing. Similarly, Koschwanez and colleagues [7] found that a significantly greater proportion of participants who performed expressive writing prior to a punch biopsy were rated as having healed wounds on day 11, as assessed from photographs, compared to those who performed control writing. However, Koschwanez et al. [7] found no significant differences between expressive and control writing in pro-inflammatory cytokine

production in peripheral blood, further emphasising the need for more research into the effects of writing on cellular processes at the wound site.

There is some evidence that expressive writing has greater effects if performed prior to wounding than afterwards, possibly due to changes in affect over time [8]. Expressive writing enhanced wound re-epithelialisation 10 days post-wound in a group who wrote expressively before the biopsy procedure compared to a group who performed control writing either before or after the biopsy [8]. The group who performed expressive writing after the biopsy showed no significant differences in healing compared to the other groups. Expressive writing caused an initial increase in negative affect and decrease in positive affect, followed by an improvement several days later.

To date, no studies have examined the effects of expressive writing on cellular processes in the healing skin. Effective wound healing requires efficient communication between different immune cells that have different functions associated with host defence, inflammation and regulation of the healing process [10]. Wound healing is commonly described as having four stages: (1) haemostasis - when vasoconstriction occurs and a blood clot forms (starts immediately), (2) inflammation – when neutrophils and macrophages reduce infection by removing foreign materials, and produce cytokines and growth factors that attract fibroblasts (starts within the hour and lasts four to five days), (3) proliferation – when the clot is replaced by granulation tissue. Fibroblasts create collagen and the extra-cellular matrix, endothelial cells promote re-epithelialisation, and keratinocytes promote re-vascularisation (takes 2-3 weeks), (4) remodelling – when type III collagen is replaced with more mature type 1 collagen (occurs over many months). Initially, neutrophils and monocytes arrive at the wound site [11]. Neutrophils are the first responders and play an important part in phagocytosis and wound debridement. After neutrophils, monocytes

migrate to the wound site and differentiate into tissue resident macrophages or dendritic cells. The role of macrophages during the inflammation phase of healing is to help promote inflammation by producing cytokines and chemokines that attract leukocytes, remove neutrophils after the early stages of inflammation, and later promote resolution of inflammation, cell proliferation and protein synthesis [12, 13].

A number of cells including macrophages, Langerhans cells and T cells, are critical for wound healing as they facilitate transitions between the aforementioned wound healing stages. Macrophages help to prevent infection by activating major histocompatibility complex (MHC) class II proteins that help the immune system recognize foreign substances through the human leukocyte antigen (HLA) system [14]. Macrophages also have an anti-inflammatory role, by decreasing immune activation by releasing anti-inflammatory cytokines (such as IL-10) and growth factors (TGF-β, VEGF and IGF-1). This inhibits the production of pro-inflammatory cytokines, stimulates angiogenesis and regulates tissue remodelling [12]. Langerhans cells (dendritic cells located in the epidermis) act as immune sentinels recognising and processing antigens [15]. During inflammation, Langerhans cells migrate from the skin to lymph nodes [16] and are repopulated in the skin by circulating monocytes after inflammation has subsided (these monocytes differentiate into Langerhans cells) [17]. Lastly, gamma-delta T cells in the dermis [18] contribute to wound healing by producing epidermal growth factors and inflammatory cytokines [19, 20] and provide protection against infection [21]. Research shows that healing is impaired when gamma-delta T cells are not present in the skin, demonstrating their importance [22].

Previous work has demonstrated that psychological factors can impact these cellular processes via a number of pathways [23, 24], affecting neutrophils and lymphocytes [25], cytokine secretion and macrophages [26], HLA expression [27], T cells [28] and Langerhans

cells [29]. It is therefore likely that expressive writing may improve healing by modulating some of these cellular processes; however, little research has been conducted to explore this theory.

In this study, healing of the 4mm biopsy was assessed by rating of epithelialisation of photographs 10 and 14 days after wounding [8]. After 14 days, participants underwent another (5mm) punch biopsy over the top of the initial 4mm wound. The removed tissue was analysed by immunohistochemistry to investigate the cellular population of the healing skin. This paper reports the analysis of this tissue in order to gain further insights into the potential effects of expressive writing on cells involved in healing.

Two main research questions are therefore addressed in this paper. Firstly, in what ways did the content (expressive vs control) and timing of writing (pre vs post wounding) alter the expression of immune cells at the wound site at day 14? Secondly, what was the association between healing at day 10, and immune cell expression at day 14? Healing was chosen at day 10 rather at day 14 because (1) there was a significant difference in healing between groups at day 10 only and (2) there was greater variation in healing at day 10 than day 14. It was hypothesised that those who performed expressive writing pre-wounding would have greater HLA expression, higher Langerhans cell infiltration, and less macrophages, neutrophils and T cells, compared to those who performed expressive writing post-wounding, or control writing at any time-point. It was also hypothesized that those who had healed wounds on day 10 would have greater HLA expression and Langerhans cell infiltration and lower levels of macrophages, neutrophils and T cells in the biopsy at day 14 than those with unhealed wounds.

1.1 Registered outcomes

Broadly defined, the primary registered outcomes of the study were speed of healing of a 4mm biopsy wound as indicated by photographs, and immunological and histological analyses of the skin. Figure 1 shows how these were further defined at the cellular level and marker level). At the level of cell markers, a total of 16 primary outcomes were registered, however we did not have enough resources to analyse all these registered outcomes. This was due to problems with sample orientation in the optimal cutting temperature (OCT) compound used to embed tissue, requiring re-orientation of the samples, making the analysis more time consuming, and resulting in costs higher than anticipated.

Analysed: As shown in Figure 1, seven primary outcomes were analysed at the marker (or day) level. These included ratings of epithelialisation from two photographs of the original 4mm biopsy wound (one on day 10 and one on day 14) (results reported in the first paper [8]). In this paper we report five histological markers from a 5mm biopsy of the healing skin at 14 days, including three cell types: Langerhans cells (marker CD207), immune (T) cell activation (markers HLA-DR and levels of CD3+ T cells), and macrophages (markers CD-68 and myeloperoxidase (MPO)). Many immune cell markers recognise more than one immune cell, for instance MPO is expressed mainly by neutrophils but also to some extent macrophages. These histological analyses give an indication of the presence and activation of cells involved in the healing process.

Not analysed: Registered outcomes that were not analysed were histological outcomes in the healthy skin from the original 4mm biopsy wound. These could have given an indication of whether expressive writing had any effect on Langerhans cells, immune cell activation and macrophages in the skin prior to wounding. Prior research has shown that the number and activation of immune cells in the skin prior to wounding can be affected by

stress and impact healing [23]. Registered outcomes related to the 5mm biopsy that were not analysed were: immune analysis of cytokines, including IL1, IL6, and TNFalpha; and matrix metalloproteinases (MMP), including proMMP9, active MMP9, and proMMP2. (Note MMP tests were performed for a subset of 48 participants only due to limited funding and results were reported in a conference abstract (no significant differences between groups)).

Therefore, although 16 primary outcome measures were planned at the marker level, only seven were fully analysed: two photographs of the 4mm wound (days 10 and 14), Langerhans cells (CD207), immune cell activation via two markers (HLA-DR and CD3+ T cells), and macrophages via two markers (CD68 and MPO) in the 5mm biopsy. Secondary outcomes were mood, perceived stress, and sleep (results reported in the first paper [8]). This second paper from the study reports the outcomes of the immunohistochemical analyses only, which were not reported in the original paper because the analyses had not been completed at the time of its publication.

2. Method

2.1. Sample

Data were collected in Auckland, New Zealand. 137 healthy participants (aged between 18 and 41) were recruited, and of these 122 received the initial wound and continued to the 14-day follow-up [8]. Participants were recruited from the University campus and local community by email, online advertisements and flyers. Potential participants were excluded if they were pregnant, had skin allergies or immunological-related health problems, were smokers, or were taking immunosuppressive medication. On enrolment into the study, participants provided written informed consent online and were

randomized to one of four intervention groups based on a 2(writing content) x 2(writing timing) randomisation ratio: “expressive writing pre-wounding”, “control writing pre-wounding”, “expressive writing post-wounding” and “control writing post-wounding”. Ethics approval was obtained from The University of Auckland Human Participants Ethics Committee (UAHPEC). Permission was obtained for storage of human tissue at the University of Bristol from the UK Human Tissue Authority.

A power analysis was conducted to calculate the number of participants required to find a difference between groups in reepithelialisation (assessed from photographs)[8] . Based on a medium effect size (Cramers V of 0.35) from a previous study[7], power of 0.80. and alpha of 0.05, 128 participants were required.

Funding was available for immunohistochemical analysis of 96 samples. 24 participants from each group were randomly selected using a random number generator. Investigators performing the immunohistochemical analysis were blind to group allocation.

2.2. Procedure

Detailed study procedures are reported elsewhere [8] but will be described here in brief. Data collection occurred between October 2014 and June 2015. Figure 2 provides an overview of the study timeline. Two weeks prior to the initial wounding appointment participants were randomized into one of the four groups. Those allocated to write pre-wounding were given intervention instructions online and asked to complete the writing task over the next three days before their appointment 14 days later.

At the first appointment all participants received a 4mm punch biopsy wound located on the inner arm, 7cm proximal to the medial epicondyle of the humerus as described elsewhere [8]. After the appointment those allocated to write post-wounding were instructed to complete the online writing task over the next three days.

10 and 14 days after wounding, the wound was photographed using an EOS 100D Canon camera (Canon Ltd., Tokyo, Japan) with a Canon Ultrasonic EF 100-mm f/2.8 Macro USM lens and Canon ringflash. A dermatologist classified each photographed wound as ‘‘healed’’ or ‘‘not healed”, with healed being defined as complete re-epithelialisation of the wound surface. The dermatologist was blinded to the group and time point the photograph was taken.

On the 14th day, a second 5mm biopsy was taken from the same site as the first biopsy, after being photographed. The biopsy was taken 14 days later as previous research has found a significant difference in healing of the tissue under the surface (using ultrasound) after expressive writing at this time point [9]. This biopsy was taken so that the healing tissue from the first wound could be removed and analysed for immunohistochemical markers. The second biopsy was 1mm larger in diameter to ensure that the entire wound site was removed. Immediately after the procedure, the tissue sample was embedded in a tin foil mould filled with FSC22 Clear Frozen Section Compound (Leica Biosystems, Melbourne), and frozen with liquid nitrogen. The sample was then stored at -80°C until analysis. Participants were given a $40 voucher as compensation for their time.

2.3. Expressive writing intervention

The expressive writing task followed standardized instructions used in previous studies [7,9]. Participants randomized to complete the expressive writing task either before or after the wound were asked to write about their “deepest thoughts and feelings about a traumatic, upsetting experience from your life.” The instructions stated that if they could not think of a traumatic experience they should write about a significant life-changing event.

Participants were asked not to write about something they had discussed in great detail with someone else.

The control instructions asked participants to write about how they spent their time. For the first session they were asked to write about the past week, for the second session they were asked to write about the past 24 hours, and for the last session they were asked to write about their plans for the upcoming week. Participants were asked to keep their writing free from emotions and only write about the facts.

Upon receiving the writing instructions, participants were asked to start writing the next day and write at home for 20 minutes a day, over three consecutive days. They were told not to worry about spelling or grammar and if they missed a day to continue with the writing task the following day. Participants used a secure online portal to complete the writing tasks, as this has been used effectively in previous research [30]. To ensure anonymity, each participant was given an individual code to log onto the online portal. The writing was saved so that it could be analysed the Linguistic Inquiry and Word Count (LIWC) [31] computer programme, which categorizes text into multiple psychologically relevant categories [32]. Five categories were analysed as per other research on expressive writing [7, 33] as they have been found to be prominent as a result of the expressive writing task; negative words (e.g., hurt and ugly), positive words (e.g., love and sweet), cognitive words (e.g., know and ought), insight words (e.g., think and consider), and personal pronouns (e.g., I and them). Participants were reminded daily by email or text to complete the writing task. At the end of each writing session, participants were asked to report how much emotion they revealed, ranging from 1 (“not at all”) to 5 (“a great deal”), to check they followed the instructions.

2.4. Measures

2.4.1. Demographic and psychological measures

Demographic measures and health behaviours were assessed at baseline two weeks prior to the first wound. Participants were asked their age, gender, weight, height, ethnicity and education level. Health behaviour data were also collected. Alcohol consumption was rated over the past three months from 1(never) to 6(everyday). On days participants did drink they were asked to rate how many drinks they had from 1(0 drinks) to 7(12 or more drinks). Participants were asked to rate how often they did physical activity for 30 minutes over an average week from 1(never) to 8(everyday). They were asked to rate their diet over the past week from 1(very poor) to 5(very good). Sleep was also assessed using the Pittsburgh Sleep Quality Index (PSQI) [34], which consists of 19 questions that are totalled. The scale demonstrated good internal reliability in this sample (*Cronbach's* α = .72). The 10 item Perceived Stress Scale (PSS) [35] was used to determine how much participants felt their lives were unpredictable, uncontrollable and stressful at baseline. Respondents were asked to indicate how often they felt a certain way over the last month on a scale from 0(never) to 4(very often). In this study, the scale had good internal reliability (*Cronbach's* α = .86). Individuals were rated as 'low stress' if they scored 25 or less (n = 50), or 'high stress' if they scored over 25 (n = 46). This split was based on the mean of the sample, which was 25.5.

2.5. Immunohistochemistry assessment

The primary outcomes reported in this manuscript relate to the analysis of cells in the second 5mm punch biopsy. This outcome quantifies different cells present in the healing skin 14 days after the initial wound. Frozen tissue samples were shipped by courier on solid carbon dioxide at -80°C to the University of Bristol. Immunohistochemical staining was

performed on the frozen skin samples embedded in freezing medium (FSC22 Clear Frozen Section Compound; Leica Biosystems, Melbourne) using mouse anti CD207 (langerin) antibody (clone 306G9, Novus Biologicals Europe, Abingdon, UK) to identify Langerhans cells in epidermis; mouse anti-CD68 (clone KP1, AbD Serotec Bio-Rad Laboratories, Inc., Hercules, CA) to identify macrophages; mouse anti-human leukocyte antigen HLA (cloneHL-39, AbD Serotec) to establish level of immune cell infiltration; mouse anti-CD3 (clone UCHT1, AbD Serotec) to identify T cells, and mouse anti-myeloperoxidase (MPO) (clone 2C7, AbD Serotec) to detect neutrophils. Non-specific antibody binding was blocked with goat serum before adding primary antibodies. Primary antibodies were detected with isotype specific biotin conjugated secondary antibodies (Jackson Immunoresearch Labs Inc., West Grove, PA). Secondary antibodies were followed by Strepavidin-biotin-horseradish peroxidase conjugates (Vector Labs). Antibody localization was performed using a peroxidase reaction with H_2O_2 and 3,3-diaminobenzidine (DAB) tetrahydrochloride (Sigma Aldrich, St. Louis, MO) as the chromogen.

Cellular infiltration for CD3, HLA, MPO, CD68 and CD207 expressing cells was assessed subjectively across the entire section using the criteria in Table 1. The scorer was blinded to group allocation. To determine subjective parameters, all sections were briefly scanned by eye to determine the level of infiltration with the different leukocyte. Then 3 representative slides from different subjective levels were manually counted to give the number of positive cells per image and then this information was used to determine the scoring system for each different leukocyte. Cellular infiltration was highly correlated at each level (dermis, epidermis base, and mid-epidermis) for CD3+ T cells, so these were summed (Cronbach's alpha = .78).

2.6. Data analysis

All data were analysed using IBM SPSS Statistics 26. To answer the first research question, mixed factorial 2 x 2 x 2 ANOVAs were conducted, with bootstrapping derived from 5000 samples, to analyse the interaction and main effects of writing content (expressive writing vs control writing) and writing timing (before vs after) as well as perceived stress at baseline (high versus low) on the immunohistochemical outcomes at day 14. Because female gender has previously been associated with faster healing, gender was entered as a covariate [36]. Perceived stress was included as a factor because expressive writing may work better for people with higher stress [37].

To manage type 1 error, two multivariate mixed ANOVA were conducted with Sidak's correction, which adjusts the significance level for multiple comparisons. One MANOVA was conducted for immune cell activation including HLA-DR and CD3+; and one MANOVA was conducted for macrophages including CD68 and MPO. For Langerhans cells, a univariate mixed ANOVA was conducted with Sidak's correction. Immunohistochemical data were not normally distributed, and therefore natural log transformations were applied and these logged values were used in the analyses. We do not plan to present any more data elsewhere.

To answer the second research question, Mann Whitney *U* Tests were conducted to analyse the associations between healing rates at day 10 and the same five immunohistochemical outcomes at day 14.

3. Results

3.1. Baseline Characteristics

Of the 96 participants included in the immunohistochemistry analysis, the age range was 18 to 41 years (*M*=23.68, *SD*=5.42). The majority of the sample were female (*N*=65,

68%) and over half the participants identified as New Zealand European (*N*=59, 61%). The rest of the sample identified as being Asian (*N*=27, 28%), Māori or Pacific Islanders (*N*=10, 10%). Table 2 shows the demographic data and baseline psychological measures for each group. There were no significant differences between groups on these measures. There were also no significant differences between the subsample of 96 participants and the larger sample of 122 with respect to any demographic or baseline characteristics.

By day 10 in the subsample, 3/24, 12/24, 6/24 and 4/24 participants had healed in the "control writing pre-wounding", "expressive writing pre-wounding", "control writing post-wounding" and "expressive writing post-wounding" respectively.

3.2. Manipulation check

In this subsample of 96 participants, those who performed the control writing task had significantly less emotion in their writing compared to those who performed the expressive writing task ($t_{(93)}$=21.29, p<.001). LIWC analysis showed that participants in the control writing groups used significantly fewer positive words ($t_{(93)}$=5.43, p<.001), negative words ($t_{(93)}$=13.13, p<.001), cognitive words ($t_{(93)}$=12.63, p<.001), insightful words ($t_{(93)}$=13.22, p<.001), and personal pronouns ($t_{(93)}$=12.46, p<.001). This indicates participants followed the writing task instructions.

3.3. Adherence to writing task

All 96 participants completed at least one writing task and 93 (97%) completed all three writing tasks. On average, participants assigned to write pre-wounding commenced writing nine days before the wound (*SD*=4.73). Participants assigned to write post-wounding, on average, commenced writing three days after the wound (*SD*=2.20). There were no significant differences between expressive and control writing groups in when they commenced writing. The writing task was completed over 5.57 days on average (*SD*=3.47),

with no significant differences between groups. 67 (70%) of the participants completed the task over the first six days or less.

3.4. *Question 1: In what ways did the content (expressive vs control) and the timing of writing (pre vs post wound) alter the expression of immune cells at the wound site at day 14?*

The adjusted means of each immunohistochemical variable for each group are shown in Figure 3. The two multivariate mixed ANOVAs showed no significant effects of writing timing, content, high or low stress, and no interaction effects for the macrophages and immune activation markers (all *ps*>.05). The covariate gender was not significant in the MANOVA for macrophages, but was significant in the MANOVA for immune activation $F(2,72) = 4.27$, $p = .018$. This indicates that neither the content nor timing of the writing intervention affected these variables in the wound. Men had higher immune cell activation in CD3+ T cells than women (p=.007).

The univariate ANOVA for Langerhans cell infiltration showed a significant main effect of writing timing ($F_{(1,85)}$=7.86, p=.006, η_p^2=.08). Irrespective of the writing content, those who wrote pre-wounding had significantly higher levels of Langerhans cell infiltration at day 14 (M=0.66, SE=0.05, 95% CI [0.55, 0.78]) than those who wrote post-wounding (M=0.45, SE=0.05, 95% CI [0.34, 0.55]). The main effect of writing content on Langerhans cell infiltration was also significant ($F_{(1,85)}$=4.00, p=.049, η_p^2=.04). Irrespective of when they wrote, those who performed expressive writing had higher Langerhans cell infiltration at day 14 (M=0.63, SE=0.06, 95% CI [0.52, 0.75]) than those who performed control writing (M=0.48, SE=0.05, 95% CI [0.37, 0.59]). There was no significant effects of gender, high or low stress levels and no significant interaction effects. These results indicate that the timing

of writing, as well as the content, may affect Langerhans cell infiltration levels 14 days after a punch biopsy wound.

3.5. Question 2: What was the association between healing at day 10, and immune cell expression at day 14?

84 of the 96 participants had healing ratings from photographs at day 10. Mann Whitney *U* tests were performed to see if there was any relationship between participants who were classified as healed on day 10 and the immunohistology data at day 14. Participants rated as healed on day 10 had significantly lower levels of macrophages (CD68) in the tissue sample than non-healed participants (healed n=25, M=2.60, SD=0.69; non healed n=59, M=2.97, SD=0.85; U=545.50, z=-1.96, p=.050). Participants rated as healed at day 10 also had significantly lower levels of CD3+ T cells (healed n=24, M=4.98, SD=1.35; non healed n=58, M=6.00, SD=2.14; U=503, z=-1.99, p=.046). There were no other significant differences in immunohistochemical outcomes.

4. Discussion

This paper examined two main research questions. First, does the content (expressive vs control) and timing (pre v post-wounding) of a writing task impact the activation of different cell types in the healing skin? The findings showed main effects of both timing and content, whereby those who wrote on either topic pre- wounding had a higher infiltration of Langerhans cells in the epidermis of the healing skin at day 14 compared to those who wrote post-wounding. In addition, those who performed expressive writing had greater infiltration than those who performed control writing, but there were no interaction effects.

The first publication from this study showed that people who performed expressive writing pre-wounding were more likely to have healed wounds at day 10 than those performing control writing [8]. This new immunohistochemical analysis suggests that the timing of writing is also an important factor when it comes to Langerhans cell infiltration, with a medium effect size. The previous paper found that by day 14, 83% of participants were rated as healed based on the photographs of the wound surface, and there were no between group differences [8]. Although most wounds were re-epithelised on day 14, underneath the surface, participants who completed either writing task prior to wounding, and those who performed expressive writing compared to control writing, had higher infiltration of Langerhans cells.

Langerhans cells are involved in the first-line defense of the epidermal barrier and are important in the inflammatory phase of healing. Previous research examining the healing of diabetic foot ulcers [38] has shown that increased Langerhans cells in the epidermis correlates with better healing. Other research has found that participants who with lower stress levels had more Langerhans cells in their skin [23]. Furthermore, faster wound healing between days 14 and 21 has been associated with more Langerhans cells in the skin at the time of wounding [23]. Therefore, these results are compatible with previous findings and are biologically plausible.

There were no other significant differences between groups in the other cells. It is possible that the current study lacked power to detect differences since it analysed only 96 of the original participants. Little immunohistochemical analysis has been performed in previous stress and wound healing research, but preliminary studies suggest that immune cells in the skin are associated with stress and healing with potentially large effect sizes (e.g. Cohens d was 0.9 for differences in HLA expression and Langerhans cells between high

stress/slow healers and low stress/fast healers in a sample of 22 participants) [23]. Expressive writing was not shown to be more effective for high stress compared to low stress individuals in this study, but this could be further explored in future research.

Wound healing is a complex process; how cells interact with each other and causality of altered patterns of healing still needs to be explored. Expressive writing is postulated to accelerate healing, and how this happens is not properly understood. Although the previous publication from this study [8], showed that expressive writing can improve overall healing (reepithelialisation), it may not cause significant changes in T cells, macrophages and neutrophils. However, further research could conduct similar analyses at a different time point in the healing process.

The second question addressed in this paper was whether there was an association between surface ratings of healing at day 10 and immune cell expression in the skin at day 14. Participants rated as healed on day 10 (regardless of content and timing allocation) had significantly lower levels of macrophages and T cells at day 14. The removal of neutrophils is an important element of resolution of inflammation and the start of the proliferative phase of healing. Macrophages are responsible for removing neutrophils in the wound by inducing neutrophil apoptosis and engaging in phagocytosis of neutrophils [39]. If neutrophils remain at the wound site for longer, they can be detrimental to healing [40].

This study has a number of limitations. The study had a larger sample size compared to other similar studies that have investigated the impact of expressive writing on wound healing [7, 9] or immunohistochemistry of healing tissue [23]. However, due to the number of groups, a larger sample may be needed to find clearer group differences. This study was limited by funding available to process the immunohistochemical samples, meaning only 96 samples were analysed. Furthermore, the study was conducted with healthy volunteers, and

thus the results cannot be generalised to clinical settings and clinical wounds. However, it is cautiously recommended that writing interventions should be conducted prior to wounding (E.g. prior to planned elective surgery) for the greatest benefits.

Another limitation is that the tissue sample was taken 14 days after the initial wounding. Therefore, changes between groups during the inflammatory phases, which occurs in the first few days after healing, could not be assessed. Future work could take the biopsy for histological analysis at an earlier timepoint. Finally, a limitation is the registration of multiple primary outcomes, some of which were described only vaguely.

Healing was measured via ratings of re-epithelialisation from photographs at day 10 and day 14, on the basis of previous research [7, 9]. Healing was not assessed on other days to reduce participant burden and due to the limited availability of the dermatologist. The study might have been stronger if the wounds were photographed on more days. Across the whole sample on day 10, 29% of wounds were re-epithelialised whereas on day 14, 83% were re-epithelialised. Possibly a photograph on day 11 would have been optimal to obtain a measure when 50% of wounds were re-epithelialised.

Future research could consider other measurements of healing (e.g. looking at percentage wound closure), rather than using a dichotomous outcome. It is difficult to rate the degree of closure based only on photographs [7]. Ultrasound offers an alternative way to analyse underlying healing but requires specialist equipment. Taking biopsies is an invasive method to assess cell activation of healing skin, and future research could consider how to measure cell activation through different stages of healing in humans. This may provide valuable information about the effects of psychological interventions on cellular infiltration and healing at different time points. Future research could also examine why some people show beneficial effects of writing on healing while others do not. Previous

research suggests that individual differences in emotional expressivity may moderate the effects of expressive writing on outcomes [41]; however, this has yet to be fully explored.

Finally, we asked participants in the pre-wounding groups to write two weeks before the initial wound. This period of time prior to wounding seemed reasonable to allow participants to experience improvements in mood after possible initial distress from writing about traumatic experiences. However, the data showed that participants wrote on a variety of days across the 14 day period prior to wounding, and our previous analysis found that those who completed the emotional writing task in the first six days were more likely to be healed at 14 days than those who completed the writing later [8]. Future research could further investigate whether writing has stronger effects when performed at different times (one to four weeks) prior to wounding. Meta-analysis of emotional disclosure interventions shows that effects on health outcomes are stronger when outcomes are measured within a month of writing compared to after a month or more [37].

4.1. Conclusion

This study suggests that both writing timing and content may affect infiltration of Langerhans cells in the healing skin 14 days after wounding. Although preliminary, these findings suggest that writing interventions should be administered prior to wounding for the best effects to be observed. Future research still needs to explore the immunological effects of writing by varying the timing of writing and healing assessments and to investigate whether these results can be replicated.

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List of Abbreviations: CD - Cluster of differentiation, HLA - Human leukocyte antigen, IL- Inter-leukin, IGF - Insulin-like growth factor, LIWC – Linguistic Inquiry and Word Count, MHC - Major histocompatibilty complex, MPO - Myeloperoxidase, TGF - Transforming growth factor, VEGF - Vascular endothelial growth factor

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Table 1

Subjective scoring for tissue sections in x20 objective field

	Tissue sections		
Score	CD3, HLA, MPO cells/field	CD68 cells/field	CD207 (Keratinocyte (Kc) ratio in the epidermis)
1	<10, sparse distribution	<50, sparse distribution	10 Kc intervals between single CD207+ cells
2	10-100, well scattered cells and up to 3 small clusters	50-100 in <30% tissue	Equal mix of 1-3Kc and 10Kc intervals between clusters of 1-3 CD207+ cells
3	>100, up to 30% tissue area infiltrated, small and large cell clusters	100-200 in 30-50% tissue	>80% epidermis has 1-3Kc intervals between frequent clusters of 1-10 CD207+ cells
4	30-50% tissue infiltrated, many large clusters	>200 in >50% tissue, presence of dense clusters	>3 areas of continuous CD207+ cells (10+), with 3-10Kc intervals
5	100% tissue infiltrated at all depths, dense distribution of positive cells	100% tissue infiltrated at all depths, dense distribution of positive cells	Nearly continuous CD207+ cells, 1-3 intervals of 1-3Kc

Table 2

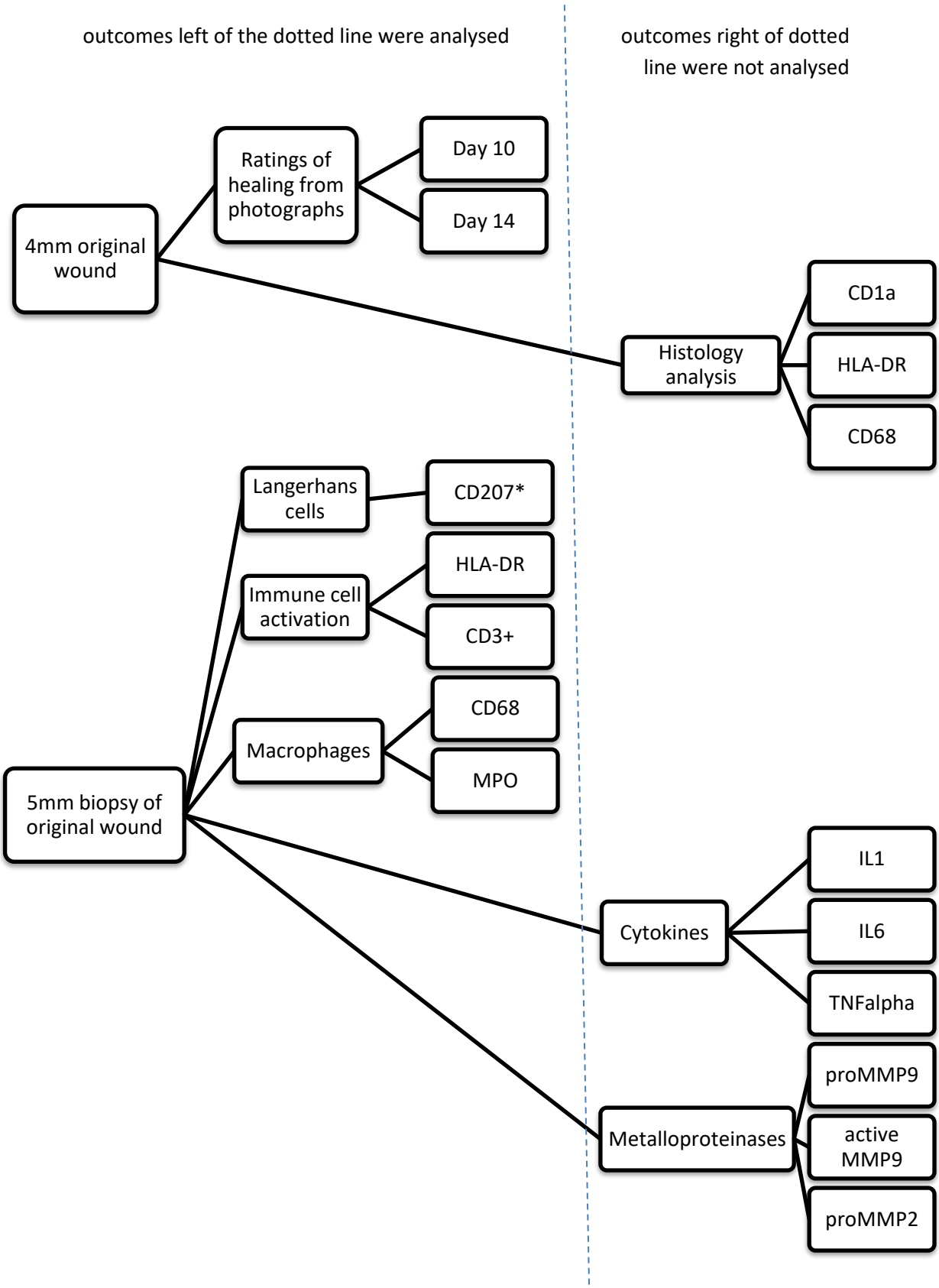
Demographics and baseline characteristics across the four experimental groups

	Control writing pre-wounding (n=24)	Expressive writing pre-wounding (n=24)	Control writing post-wounding (n=24)	Expressive writing post-wounding (n=24)	*p*
Demographics					
Age, *M(SD)*	22.96(4.21)	24.17(6.77)	24.83(6.49)	22.7(3.54)	.498[a]
Gender:					.488[b]
Women, *N*(%)	14(58%)	19(79%)	16(67%)	16(67%)	
Men, *N*(%)	10(42%)	5(22%)	8(33%)	8(33%)	
Ethnicity:					.815[b]
European, *N*(%)	14(58%)	16(67%)	15(63%)	14(58%)	
Asian, *N*(%)	5(21%)	8(33%)	6(25%)	8(33%)	
Māori or Pacific Island, *N*(%)	5(21%)	0(0%)	3(12%)	2(8%)	
Health Behaviours					
No. of times alcohol consumed over past three months:					.706[b]
None, *N*(%)	5(21%)	6(25%)	2(8%)	4(17%)	
Several times per month, *N*(%)	13(54%)	9(38%)	16(67%)	13(54%)	
Several times per week, *N*(%)	6(25%)	9(38%)	6(25%)	7(29%)	
Exercise per week:					.523[b]
0-1 times a week, *N*(%)	2(8%)	3(12%)	1(4%)	1(4%)	
2-4 times a week, *N*(%)	15(63%)	15(63%)	13(54%)	13(54%)	
5-7 times a week, *N*(%)	7(29%)	6(25%)	10(42%)	10(42%)	
Sleep (PSIQ), *M(SD)*	5.45(2.39)	4.82(2.07)	5.59(3.05)	5.22(2.35)	.802[a]

Diet (rating out of 5; 1= very poor, 5= very good), *M(SD)*	3.67(0.67)	3.50(0.80)	3.00(0.60)	3.75(0.75)	.452[a]
Psychological measures					
Perceived Stress Scale (PSS), *M(SD)*	25.63(7.64)	24.58(6.54)	25.29(6.58)	26.71(5.81)	.738[a]

Note: [a]One way ANOVA[a]; [b]Chi square

Fig. 1. Primary outcomes arranged in a hierarchical structure. The boxes right of the dotted line contain registered outcomes that were not analysed.



Note: * CD207 was used as marker for Langerhans cells rather than CD1a, as it's newer and more specific.

Fig. 2. Flow chart of study timeline for each group

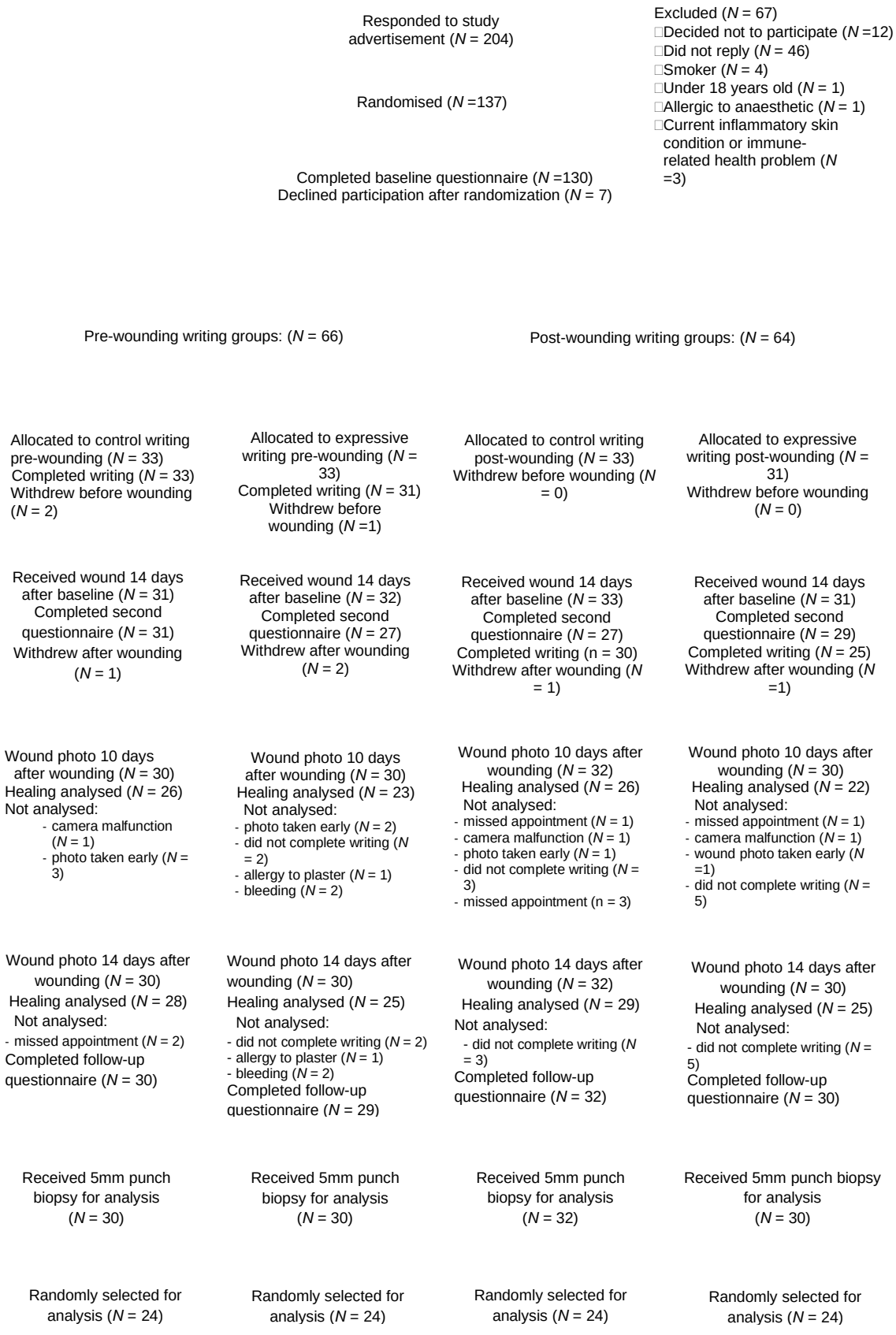


Fig. 3. Comparisons of macrophages (CD68 and MPO), immune cell activation (HLA and levels of CD3+ T cells, and Langerhans cells (CD207) between groups. Columns show estimated marginal means of natural log scores with standard error bars, with gender as a covariate. There were only significant effects for Langerhans cells.