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Neuroimmune responses following joint mobilisation and manipulation in people with persistent neck pain: A protocol for a randomised placebo-controlled trial

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Contributorship statement

All authors were involved in the design of the study and the acquisition of the research funds. ILS drafted the initial versions of the article. All authors critically revised the various drafts and approved the final version.

Competing interests

The authors have no known conflict of interest and have no commercial interest in this study.

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Data sharing statement

Data are available upon reasonable request.

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ABSTRACT

***Introduction**:* Joint mobilisation and manipulation often results in immediate pain relief in people with neck pain. However, the biological mechanisms behind pain relief are largely unknown. There is preliminary evidence that joint mobilisation and manipulation lessens the upregulated neuroimmune responses in people with persistent neck pain.

***Methods and analysis**:* This study protocol describes a randomised placebo-controlled trial to investigate whether joint mobilisation and manipulation influence neuroimmune responses in people with persistent neck pain. People with persistent neck pain (N=100) will be allocated, in a randomised and concealed manner, to the experimental or control group (ratio 3:1). Short-term (i.e., baseline, immediately after and two-hours after the intervention) neuroimmune responses will be assessed, such as inflammatory marker concentration following *in-vitro* stimulation of whole blood cells, systemic inflammatory marker concentrations directly from blood samples, phenotypic analysis of peripheral blood mononuclear cells, and serum cortisol. Participants assigned to the experimental group (N=75) will receive cervical mobilisations targeting the painful and/or restricted cervical segments and a distraction manipulation of the cervico-thoracic junction. Participants assigned to the control group (N=25) will receive a placebo mobilisation and placebo manipulation. Using linear mixed models, the short-term neuroimmune responses will be compared 1) between people in the experimental and control group, 2) within the experimental group, between people who experience a good outcome and those with a poor outcome. Furthermore, the association between the short-term neuroimmune responses and pain relief following joint mobilisation and manipulation will be tested in the experimental group.

Ethics and dissemination*:* This trial is approved by the Medical Ethics Committee of Amsterdam University Medical Centre, location VUmc (Approval number: 2018.181).

Trial registration number and status*:* The study protocol is registered at trialregister.nl with study ID: NL6575 registered on 18-01-2018; Recruitment commenced 26 February 2019. All data are anticipated to be collected by January 2022, when data analysis and interpretation are anticipated to commence.

Strengths and limitations of this study

- This study provides insight in the interplay between joint mobilisation and manipulation, neuroimmune responses, and pain relief in people with persistent neck pain.
- By adding a placebo-control group, possible working mechanisms of joint mobilisation and manipulation on neuroimmune responses may be revealed.
- The interventions will be delivered by two musculoskeletal physiotherapists, which may limit the generalisability.
- Due to the small control group, it is not feasible to divide the control participants according to outcome.

1. INTRODUCTION

The disruption of the bidirectional communication pathways between the central nervous system and the immune system may play an important role in persistent pain.(1) Over the last two decades, it has become apparent that neuroimmune crosstalk is present in musculoskeletal pain, and may play a mediating role in the transition from acute to persistent pain.(1) For people with persistent neck pain, aberrant neuroimmune responses may be present, such as systemically elevated levels of inflammatory markers.(2, 3) These increased neuroimmune responses may be relevant to understand and manage persistent spinal pain.(3) A growing body of literature suggests that these neuroimmune responses are associated with pain intensity,(4-6) disability(7) and recovery,(8) and can be influenced by musculoskeletal physiotherapy, such as joint mobilisation and manipulation,(9-11) nerve mobilisation(12, 13) and exercise.(14-16)

Several meta-analyses indicate that musculoskeletal physiotherapy for people with spinal pain may provide immediately pain relief and improvements in functional activities compared to no treatment, placebo or other treatments.(17-19) Nevertheless, unravelling the mechanism of how joint mobilisation and manipulation results in pain relief remains an area for further investigation.(20, 21) There are various explanations of how joint mobilisation and manipulation might cause pain relief, including neurophysiological,(22, 23) neuromuscular,(20) neuroimmune(24, 25) and non-specific responses.(26)

Recent studies suggest a possible neuroimmune-mediated mechanism of pain relief following joint mobilisation and manipulation.(9-11) For example, a reduction in systemic inflammatory marker concentration directly from blood samples(9, 11) and a reduction in inflammatory marker concentration following *in-vitro* stimulation of whole blood cells(10, 27) were found immediately following the intervention. These studies have however

important methodological limitations, such as inclusion of healthy participants,(27) modest sample sizes,(9, 10) a narrow selection of inflammatory markers,(9-11) lack of correction for potential confounding variables,(9, 10, 28) and lack of a placebo-control group.(9, 10) Therefore, we will conduct an adequately powered, placebo-controlled randomised clinical trial in people with persistent neck pain, which will evaluate a broad range of inflammatory markers. The purpose of this paper is to describe the study protocol to investigate the short-term effects of joint mobilisation and manipulation on neuroimmune responses in people with persistent neck pain.

2. METHODS

This manuscript followed the guidelines for clinical trial protocols (SPIRIT statement),(29) for reporting randomised trials (CONSORT statement),(30) and for intervention description and replication (TIDieR checklist).(31)

Aim

The overall aim of this clinical trial is to gain insights in the relation between short-term neuroimmune responses following joint mobilisation and manipulation and pain relief in people with persistent neck pain. The specific aims are: 1) to compare the short-term neuroimmune responses between the experimental and control group; 2) to compare the short-term neuroimmune responses of those in the experimental group with a good outcome (i.e., immediately pain relief) with those in the experimental group with a poor outcome; and 3) to assess the association between short-term neuroimmune responses and pain relief in the experimental group.

Study design and setting

The study is a placebo-controlled randomised trial with follow-up at three time points: baseline, immediately, and two-hours and two-days following the intervention (Fig. 1). Participants will be recruited from GP clinics, primary care physiotherapy practices and outpatient services (neurology and orthopaedic departments) at secondary care hospitals. Data are anticipated to be collected between February 2019 and January 2022, when data analysis and interpretation are anticipated to commence.

Selection criteria

Individuals meeting the following inclusion criteria are eligible to participate: age: 18-65 years; non-specific neck pain for at least six weeks(32) with a minimum pain intensity of 40/100 on a visual analogue scale (VAS), and a sufficient speaking and reading level of the Dutch language to complete the study. Exclusion criteria are contra-indications for cervical mobilisation or cervico-thoracic manipulation,(33, 34) pregnancy or less than 9 months postpartum, contra-indications for venipuncture (e.g., phlebitis), treatment for the current neck pain episode during the preceding two weeks, taken corticosteroids or cytokine modulatory medication (e.g., methotrexate, infliximab) in the preceding 6 weeks, use of botulinum toxin (Botox) injection during the preceding 3 months, non-steroid anti-inflammatory drug medication within the past 7 days (e.g., diclofenac, ibuprofen, naproxen), long-distance flight within the past 7 days, ongoing shift work, having a known comorbid condition with immune/endocrine malfunction (e.g., ankylosing spondylitis, Crohn's disease, sarcoidosis, Cushing syndrome, cancer, diabetes), medical red flags suggestive of serious pathology,(35, 36) and a diagnosed psychological condition (e.g., clinical depression).

Consecutive participants who meet all selection criteria and are willing to participate will be admitted to the study. All participants will provide written informed consent prior to participation. Initial screening for eligibility will be conducted via telephone calls.

Randomisation, concealed allocation and blinding

Block randomisation will be used to allocate participants to the experimental or control group with an allocation ratio of 3:1 (experimental : control). A computer random number generator will create block sizes of 4 and 8 participants. To conceal the allocation sequence, an independent person not involved in the study will assign eligible people to the groups on the day the participant will enrol in the study. Blood samples will be coded to blind the research assistant and laboratory investigators to the study groups. The participant, research assistant and the investigator who includes the participants will be blinded for group assignment. The treating clinicians, research assistant and laboratory investigators will be unaware whether participants experienced a good outcome or not. All laboratory and data analyses will be performed by blinded investigators.

Interventions

Experimental intervention

Spinal mobilisation will consist of low-velocity, low-amplitude mobilisations at the painful cervical segmental levels (Fig. 2 – Panels A-C); spinal manipulation will consist of a high-velocity, low-amplitude distraction manipulation at the cervico-thoracic junction (Fig. 2 – Panel D).(37) These techniques aim to restore motion and reduce pain. They are commonly used and are conform to the Dutch guidelines for musculoskeletal physiotherapy for treating neck pain.(35) All interventions will be performed by two musculoskeletal physiotherapists with more than 5 years of relevant clinical experience.

Cervical mobilisation

Painful and restricted cervical segments will be identified by passive side-bending of the neck targeting each segmental level separately.(38) Reproduction of the participant's pain will be considered to identify the involved level(s). The inter-tester reliability for these tests is fair to substantial.(38, 39)

Depending on the identified painful or restricted spinal levels, the treating clinician may select from different mobilisation techniques: mobilisation targeting the atlanto-axial segment (Fig. 2 – Panel A); segmental zygapophyseal joint mobilisation (C2 to C7) (Fig. 2 – Panel B) and occipital-atlanto-axial joint mobilisation (Fig. 2 – Panel C). Three series of oscillations (~1Hz) will be applied for 30 seconds; with 30 seconds rest in between the series.

Cervico-thoracic junction distraction manipulation

Irrespective of the level of their neck pain, all participants will receive a distraction manipulation of the cervico-thoracic junction (Fig. 2– Panel D).(40) If there is no audible cavitation sound during the first attempt, the manipulation will be repeated once.

Control (placebo) intervention

The control group will receive a placebo mobilisation and placebo manipulation. Procedures, including the instructions, will be identical as for the experimental intervention, except that the clinician will only apply hand contact and no pressure or movement will occur. Participants will be informed that an audible popping sound may or may not occur, and that this sound is not necessary to restore motion and reduce pain.

The credibility of a control intervention can interact with participant expectations in complex ways.(41) To account for differences in intervention expectations, participants will

indicate the extent to which they agree (using a four-point Likert scale) with four statements regarding their intervention expectations (Table 1). These statements will be presented before the delivery of the experimental and control intervention.(42)

Based on the short-term changes in pain intensity score (i.e., immediately and two-hours following the intervention), participants in the experimental group will be categorised into those with a good outcome (≥50% improvement in pain intensity at both time points), a poor outcome (≤20% improvement in pain intensity score at both time points) or an unclear outcome (not fitting the criteria for a good or poor outcome).(43) Based on these cut-off scores, we anticipate to have a minimum of 25 participants in both the good outcome and poor outcome group. If our *a-priori* determined minimum of 25 participants in either group is not achieved, the good outcome group and the poor outcome group will be supplemented with respectively the best responders and poorest responders from the uncertain outcome group in order to obtain 25 participants in both groups.

Outcomes

A broad range of neuroimmune responses will be monitored: a) inflammatory marker concentration following *in-vitro* stimulation of whole blood cells, b) systemic inflammatory marker concentrations directly from blood samples, c) phenotypic analysis of peripheral blood mononuclear cells and d) *ex-vivo* serum cortisol (Table 1). To create an inflammatory profile,(44) a range of pro-inflammatory and anti-inflammatory markers will be used. Ex-vivo serum and supernatants after stimulation will be stored at minus 80°C and will be analysed upon completion of data collection. The laboratory methodology and sample handling prior to stimulation will be tightly monitored and reported, because inconsistency in interlaboratory methodology and reporting impairs interpretation, comparability and reproducibility.(45)

Primary outcomes

The primary outcomes are the short-term (i.e., immediately and two-hours following the intervention) differences in interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) following *in-vitro* stimulation of whole blood cells. These cytokines will be determined using Meso Scale Discovery (MSD, Maryland United States) at baseline, immediately and two-hours following the intervention. These cytokines are selected because previous research has indicated that those cytokines might play a role in spinal pain.(11, 27, 46-48)

To induce cytokine production, whole blood cultures will be stimulated for 24 hours with lipopolysaccharide (LPS) from Escherichia coli O55:B5 (Sigma-Aldrich Chemie GmbH, Schnelldorg, Germany) at a concentration of 1 nanogram LPS/millilitre whole blood (ng/ml) and 10 microgram LPS/millilitre whole blood (µg/ml) at 37°C in a humidified 5% CO_2 incubator. At baseline (Fig. 1) blood samples for neuroimmune measurements (one sodium heparin vacutainer without gel and one serum vacutainer without gel for each time point) will be drawn between 8:00 and 9:00 AM.(49) The cytokine levels will be determined using a custom-made U-plex MSD and expressed in picogram/millilitre (pg/ml). The entire blood stimulation procedure and MSD will be performed by an experienced laboratory technician at Amsterdam University Medical Centre, location VUmc, Department of Clinical Chemistry, Medical Immunology Laboratory.

Secondary outcomes

Several additional neuroimmune responses will be quantified as secondary outcomes at various time points (Table 1).

The levels of interleukin-1 receptor antagonist (IL-1RA), interleukin-4 (IL-4), interleukin-10 (IL-10), c-c motif chemokine ligand 2 (CCL2), c-c motif chemokine ligand 3

(CCL3) and c-c motif chemokine ligand 4 (CCL4) will be determined following *in-vitro* stimulation of whole blood cells.

Systemic inflammatory markers directly from blood samples (tumor necrosis factor – receptor antagonist II (TNF-RII), IL-1β and IL-1RA) will be measured using multianalyte assay Ella (R&D systems, Minneapolis, United States) and high-sensitive c-reactive protein (hsCRP), using Roche/Hitachi cobas c systems (Indianapolis, United States).

Phenotypic analysis of peripheral blood mononuclear cells will be determined. The absolute number of lymphocyte subsets (NK cells, B-cells, $CD4^{+}$ and $CD8^{+}$ T-cells and $CD25^{hi}$ regulatory T-cells), monocytes, as well as activation status of these cells, HLA-DR and TLR-4 expression, will be determined by 10-color flowcytometry (FCM, Gallios Flow Cytometer, Beckman Coulter, Indianapolis, United States; Analyse software: Kaluza). Differences between all groups in serum cortisol concentration will be determined using conventional electrochemiluminescence immunoassay (ECLIA) from Roche (Cobas Cortisol, 2nd generation, Indianapolis, United States) in agreement with the manufacturer's protocol.

Procedures

Once consent is obtained, baseline measurements will be taken (Fig. 1). At baseline, participants will undergo physical tests to determine pain characteristics, physical functioning and body composition (Table 2 & Table 3). After this, participants will complete an electronic survey to collect sociodemographic and clinical information (Table 1) and intervention expectations (Appendix A). Participants will then undergo one venipuncture from the cubital vein to fill two vacutainers which will be used to quantify the neuroimmune responses (Table 1). Collection of all baseline data will take 30-45 minutes and will take place at the Amsterdam University Medical Centre, location VUmc, or at a participating primary care physiotherapy practice, under the supervision of a research assistant.

Participants will then be randomly allocated to the experimental and control group, and treated accordingly. Immediately and two-hours following the intervention, participants will undergo another venipuncture to fill two vacutainers. Between the immediate and two-hours follow-up measures, questionnaires will be completed to collect psychosocial information such as sleep, disability and kinesiophobia (Table 2).

Immediately and two-hours following the intervention, participants will undergo physical tests (Fig. 1) and will rate their pain intensity on a VAS. Two-hours following the intervention, participant will rate their perceived recovery on a 7-point Global Perceived Effect scale (GPE) (Table 2). Two-days following the intervention, participants will receive an electronic survey regarding potential adverse events, GPE and pain intensity. Figure 1 shows the planned flow of participants through the study.

Sample size

Based on the sample size calculation(50) (longitudinal analysis; three time points (baseline, immediately follow-up, two-hours follow-up) with 80% power to detect a mean difference of 550 (SD 933) for TNF-α levels with a 0.05 two-sided significance level, correlation of 0.6 among repeated measures, ratio between groups of 0.25, a total sample size of 91 is needed.(27) Allowing for a drop-out rate of ~10%, a total sample size of 100 participants is required.

Statistical analyses

Data will be checked for normality by the Kolmogorov-Smirnov test and visual inspection of Q-Q plots, box plots and histograms. In case of no normality of data, the data will be log transformation. Data will be presented as means with standard deviations unless otherwise noted. For the analyses, statistical significance will be set at $p<0.05$. Intention-to-

treat analyses using mixed models will be performed to analyse differences between the experimental group and control group. Linear mixed model analyses with fixed factor (time), covariate (group) and interaction (time*group) will be used to detect differences between the groups at the three time points (baseline, immediately follow-up, two-hours follow-up) for TNF-α and IL-β following *in-vitro* stimulation of whole blood cells. A random intercept will be selected to account for the correlated nature of multiple measurements from the same participant. The regression coefficient (B), p-value and confidence intervals (95%CI) will be computed for the crude models, as well as for the adjusted models.(28, 51) Linear regression analysis will be used to test for differences in phenotypic analysis of peripheral blood mononuclear cells and cortisol between the experimental and control group and of those in the experimental group with a good outcome (i.e., immediate pain relief) with those in the experimental group with a poor outcome.

Adverse events

Serious and non-serious adverse events related to the experimental and control intervention, and all other aspects of the study, will be documented. At the three post-intervention time points, potential adverse events will be recorded using an online survey. Adverse events will be followed-up as needed by an independent clinician. Depending on the nature of the event, participants may be referred to a GP or a medical specialist, and additional tests or procedures may be proposed. The experimental intervention has been shown to be safe(11, 27) and it is considered unlikely that serious adverse events due to the interventions will occur. Therefore, installing a data monitoring safety board was not requested by the Ethics Committee.

Patient and public involvement

A panel of four people with persistent neck pain co-developed and evaluated the study design, research questions, choice of experimental and control intervention, and burden of study participation for the participants. Two of these people and two representatives from the public reviewed the Patient information letter and their feedback was used to improve the letter.

Data management and monitoring

The data will be collected at the Department of Rehabilitation of the Amsterdam University Medical Centre, location VUmc, and/or in physiotherapy practices. The collected data will be securely stored at Vrije Universiteit Amsterdam, Faculty of Behavioural and Movement Sciences. All data are de-identified by using unique participant ID numbers in such a way that the data cannot be traced back to the individual participants without the key. The participants code will exist of a random code of three numbers. The electronically key connecting participant names with codes will be kept in a secure location in the principal investigator's office. The key will be kept for six months after the final publication, and will then be destroyed. Data will be stored in a de-identified manner for fifteen years after the final publication.

Role of funding source

This study is funded by the Dutch Association for Manual Therapy (NVMT, grant ID. Top-down_2018) and by the Faculty of Behavioural and Movement Sciences (grant ID. Lab Fund_2019) of Vrije Universiteit Amsterdam. The MSG Science Network (https://www.msg-sciencenetwerk.nl/) (grant ID. N/A)will support participant recruitment. The funding sources have no role in the study design and will not have any roles in data collection, analysis and interpretation of the data, nor in the reporting of the results.

Ethics and dissemination

The study is registered at trialregister.nl with study identification NL6575. The results of the study will be published in peer-reviewed journals and disseminated at conferences, in newsletters and social media. The trial is approved by the Medical Ethics Committee of Amsterdam University Medical Centre, location VUmc (Approval number: 2018.181). All procedures will be conducted in accordance with the Declaration of Helsinki.(52) Amendment to this protocol will be submitted for approval to the Medical Ethical Committee and deviations from the protocol will be reported to the trial registration.

3. DISCUSSION

There is considerable debate in the literature regarding the possibility of meaningful neuroimmune-mediated pain relief following joint mobilisation and manipulation.(2, 7, 53, 54) We described a protocol for a randomised placebo-controlled study that will assess potential neuroimmune-mediated pain relief following joint mobilisation and manipulation in people with persistent neck pain. The aim of this study is to gain insights in the relation between changes in neuroimmune responses and pain relief, rather than in the clinical efficacy or effectiveness of joint mobilisation and manipulation for people with persistent neck pain.

Recent data suggest that the production of pro-inflammatory cytokines is higher and production of anti-inflammatory cytokines is lower in patients with persistent-pain compared to healthy people following *in-vitro* stimulation of whole blood cells.(44) Additionally, a specific, coordinated inflammatory processes may be important for patient recovery.(3) Contrary to the other studies we are aware of that measured neuroimmune responses following joint mobilisation and manipulation,(7, 44, 55) we will assess a comprehensive

range of inflammatory markers. Our approach to measure pro-inflammatory cytokines and their antagonists provides insight into the activation of immunocompetent cells.(56)

We believe the design of our study allows to assess the specific effects of joint mobilisation and manipulation on neuroimmune responses. For instance, rather than comparing the joint mobilisation and manipulation with a wait-and-see approach, we will compare responses with a placebo-control intervention that resembles joint mobilisation and manipulation. Additionally, the verbal instructions between the experimental and control groups will be comparable and standardised, which reduces differences in intervention efficacy due to non-specific intervention effects.(57) Differences in verbal instructions have been shown to be associated with differences in endocrine responses following joint manipulation in people with neck pain.(58) Finally, we will record the participant's intervention expectations and beliefs regarding joint mobilisation and manipulation as a treatment method to alleviate neck pain.(42)

Previous research revealed a non-linearity of the VAS to measure pain intensity, that responsiveness varies along the spectrum of pain intensity and the importance of taking baseline pain into account when evaluating change scores.(43, 59, 60) Consequently, categorising good, unclear and poor outcome using raw data, or change scores in general, are invalid as these will either underestimate or overestimate true change.(60) To overcome this problem, we follow the initiative on methods, measurement and pain assessment in clinical trials (IMMPACT) recommendation to identify those with a good, poor outcome, or unclear outcome.(43)

Besides the strengths, the proposed study has some potential limitations. First, we assume a linear association between neuroimmune responses and musculoskeletal pain. A linear association between neuroimmune responses and musculoskeletal pain is a prerequisite for the justification of the statistics proposed in this protocol. However, one study suggests

that an initial threshold of neuroimmune responses might be required, which would suggest a non-linear relationship between neuroimmune responses and musculoskeletal pain.(61) In that study, elevated IL-6 levels were only present in the group of people with pain > 40/100 VAS compared to control.(61) Therefore, a minimal pain intensity of 40/100 on the VAS will be a prerequisite for participating in this study.

Another limitation is that only a single session of joint mobilisation and manipulation will be provided together with a short follow-up. While a single session of joint mobilisation and manipulation may induce a pain-relieving effect,(17) the clinical relevance of immediately pain relief is unclear. Nonetheless, our aim is not to examine the efficacy of joint mobilisation and manipulation but rather to understand the biological mechanisms behind pain relief following joint mobilisation and manipulation. In studying the mechanism of action, a short follow-up has the advantage that potential confounding variables can be controlled, such as food intake, stress, physical exercise and health status.

Contributorship statement

All authors were involved in the design of the study and the acquisition of the research funds. ILS drafted the initial versions of the article. All authors critically revised the various drafts and approved the final version.

Competing interests

The authors have no known conflict of interest and have no commercial interest in this study.

Funding

This study is funded by the Dutch Association for Manual Therapy (NVMT, grant ID. Top-down grant_2018) and by the Faculty of Behavioural and Movement Sciences (grant ID. Lab Fund_2019) of Vrije Universiteit Amsterdam.

Data sharing statement

Data are available upon reasonable request.

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Figure 1: Anticipated flow of the study

Abbreviations: GPE: global perceived effect; VAS: visual analogue scale

Figure 2. Spinal mobilisation and manipulation techniques.

Depending on the identified painful segmental levels, the clinician can select from different cervical mobilisation techniques (A-C); For techniques A-C, the participant will be seated on a chair, leaning against the upper leg or shoulder of the clinician. **Panel A***:* Mobilisation targeting the atlanto-axial joints. The cervical segments below the second cervical vertebrae are submaximal rotated and lateroflexed. With the clinician's hypothenar region of the hand over the structures overlying the arcus of the first vertebrae, the clinician moved the head further in rotation.(39) **Panel B:** Segmental zygapophyseal joint mobilisation (C2 to C7; the image shows the technique for C3-C4). First, the occipital-atlanto-axial joint is maximally rotated in the direction of the facet joint being mobilised. Subsequently, the head is moved to extension, ipsilateral lateroflexion and rotation until pressure from the thumb is felt. This technique is repeated on the lower level until the painful cervical segment is reached (C3-C4). Next, on the painful cervical segment, pressure will be given in a cranio-ventral direction. (39) **Panel C:** Mobilisation technique targeting the occipital-atlanto-axial joints. The clinician's hypothenar region is placed against the mastoid process. C2 to C7 are submaximally locked in flexion, rotation and lateroflexion. The head is then moved in a medio-caudal direction.(39) **Panel D:** Spinal manipulation technique targeting the cervico-thoracic junction. The participant will be seated on a treatment table. The height of the table will be adjusted to the level of the clinician's abdomen. The participant's hands will be placed on the back of their head (with one hand placed over the other hand, rather than with interlocking fingers), and with the shoulders slightly retracted. The clinician's hands will be placed over the hands of the participant, with the clinician's forearms ventral to the shoulder of the participant. Then, a high-velocity, low-amplitude movement will be applied in a dorsal-cranial direction.(39) Green arrows represent the direction of the mobilisation (Panel A-C) or manipulation (Panel D).

Table 1: Overview of the neuroimmune responses

Domain	Neuroimmune parameters	Timing of measurements			
		T0	T1	T2	T3
Systemic inflammatory marker directly from blood samples[a]	TNF-α, TNF-RII, IL-1β, IL-1RA, hsCRP[b]	√	√	√	-
Inflammatory marker concentration after *in-vitro* stimulation of whole blood cells[c]	TNF-α, IL-1β, IL-1RA, IL-4, IL-10, CCL2, CCL3, CCL4	√	√	√	-
Ex-vivo serum cortisol[d]	Cortisol	√	√	-	-
Phenotypic analysis of peripheral blood mononuclear cells[e]	$CD45^+$, $CD3^+$, $CD4^+$, $CD25^{hi}$,$CD8^+$,$CD56^+$, $CD19^+$, $CD14^+$, HLA-DR, TLR-4	√	-	√	-

a) Measured using multianalyte assay Ella (R&D systems, Minneapolis, United States)
b) Cardiac C-Reactive Protein (Latex) High Sensitive using Roche/Hitachi cobas c systems.
c) Stimulated for 24 hours at 37°C, in a humidified 5% CO_2 incubator, with lipopolysaccharide (LPS) from *Escherichia coli O55:B5* at a concentration of 1ng/ml and 10µg/ml. Determined using a custom-made U-plex (MSD, Maryland, United States)
d) Using conventional electrochemiluminescence immunoassay (ECLIA), Roche (Cobas Cortisol, 2nd generation).
e) Determined by 10-color flowcytometry (FCM): CD45+ = General Leukocyte marker; CD3+ = T-cell marker; CD3+CD4+ = CD4+ T-helper marker; CD3+CD4+CD25hi = T-regulator cell marker; CD3+CD8+ = Cytotoxic T-cell marker; CD3-CD56+ = Natural Killer cell marker; CD19+ = B-cell marker; CD14+ = monocyte marker; HLA-DR = activation marker for T-cells and monocytes; TLR-4 = Toll-like receptor 4 marker.

Abbreviations: T0: baseline; T1: immediately following the intervention; T2: two-hours following the intervention; T3: two-days following the intervention; TNF-α: Tumor Necrosis Factor-α; TNF-RII: Tumor Necrosis Factor Receptor Antagonist 2; IL-1β: Interleukin-1β; IL-1RA: Interleukin-1 receptor antagonist; hsCRP: High sensitive C-Reactive Protein; IL-4: Interleukin-4; IL-10: Interleukin-10; CCL2: c-c-motif chemokine ligand 2; CCL3: c-c-motif chemokine ligand 3; CCL4: c-c-motif chemokine ligand 4; CD: Cluster of Differentiation

Table 2: Self-reported questionnaires and physical tests

Domain	Self-reported questionnaires	Timing of measurements			
		T0	T1	T2	T3
Disability	Neck Disability Index (NDI)[a]	-	√	-	-
Perceived effect	Global Perceived Effect (GPE)[b]	-	-	√	√
Fear of movement	Tampa Scale of Kinesiophobia[c]	-	√	-	-
Type of pain	PAIN Detect Questionnaire (PDQ)[d]	-	√	-	-
Type of pain	Central Sensitisation Inventory (CSI)[e]	-	√	-	-
Depression, Anxiety, Stress	Depression Anxiety Stress Scale (DASS21)[f]	-	√	-	-
Physical activity	International Physical Activity Questionnaire (IPAQ)[g]	-	√	-	-
Catastrophising	Pain Catastrophising Scale (PCS)[h]	-	√	-	-
Sleep Quality	Pittsburgh Sleep Quality Index (PSQI)[i]	-	√	-	-
Pain Intensity	Visual Analogue Scale (VAS)[j]	√	√	√	√
Mental health	Mental health inventory (MHI-5)[k]	√	-	-	-
Domain	**Physical tests**	**Timing of measurement**			
		T0	**T1**	**T2**	**T3**
Range of motion	Cervical Range of Motion (CROM)[l]	√	√	√	-
Pain intensity	CROM-VAS test[m]	-	√	√	-
Quantitative sensory testing	Pressure Pain Threshold (PPT)[n]	√	√	√	-
Quantitative sensory testing	Wind-up ratio[o]	√	√	√	-

a) The Dutch version of the NDI is a valid and responsive measure of disability.(61)
b) The GPE is a validated and reliable tool to assess health transitions in patients with musculoskeletal disorders.(62)
c) Preferred self-administrated questionnaire to asses fear of movement in musculoskeletal pain.(63)
d) Persistent pain will be categorised in two-mechanism based groups: nociceptive and neuropathic pain using the PDQ. The PD-Q is a reliable screening tool with high specificity.(64)
e) The Dutch Central Sensitization Inventory (CSI) has good internal consistency, good discriminative power and excellent test-retest reliability. A cut-off score of 40/100 provides a sensitivity of 81% and specificity of 75%.(65)

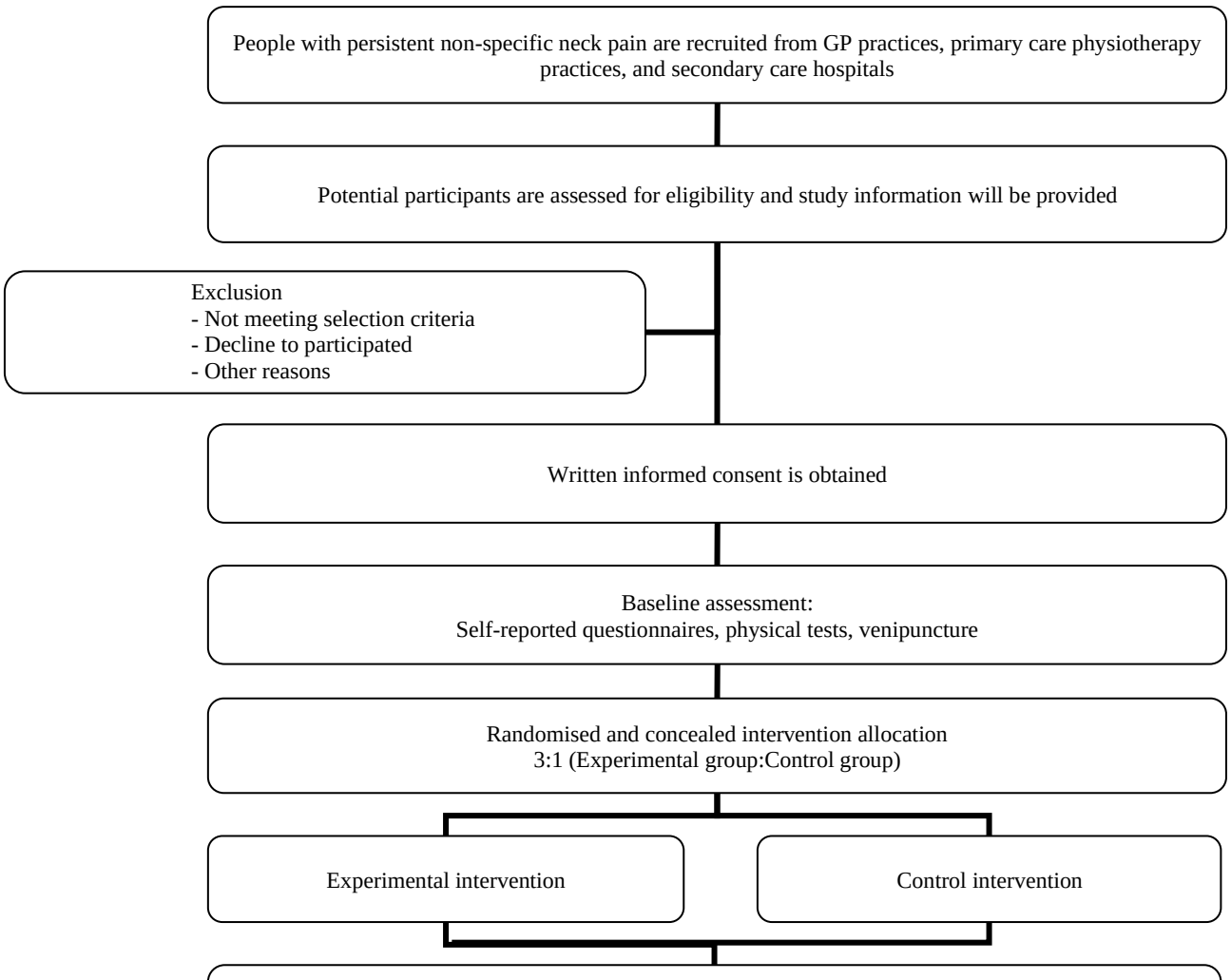
f) Preferred self-administrated questionnaire to assess depression, anxiety and stress in musculoskeletal pain.(63, 66)
g) Expressed in 1000 metabolic equivalent minutes per week (Dutch-language version).(67) The IPAQ has good reliability (intraclass correlation coefficient [ICC] = 0.70-0.96) and moderate validity (r = 0.36-0.49) of the IPAQ compared with an accelerometer.(68)
h) Preferred self-administrated questionnaire to assess pain catastrophising in musculoskeletal pain.(63)
i) Score above 5 yield a sensitivity of 89.6% and specificity of 86.5% in distinguishing good and poor sleepers.(69)
j) The reliability and validity of the VAS as a measure of pain for neck pain patients is good.(70)
k) General psychological status will be assessed using the MHI-5.(71) A higher score indicates better mental health. Cronbach's alpha for the MHI-5 scale is 0.85.(72)
l) The CROM is a clinically reliable tool to measure active cervical range of motion people with neck pain and healthy participants.(73)
m) This novel test consists of two parts. In Part 1, the participant is asked to perform maximal active right and left cervical rotation and the degrees of rotation are reordered using the CROM device. In this position, the pain intensity is measured with the VAS following intervention. After the intervention, Part 2 of the test is performed . The participant is again asked to actively rotate (left and right) to the same position as in Part 1 and the pain intensity is recorded. The difference on VAS scores is the outcome of the CROM-VAS test.
n) Pressure algometry over the cervical spine has shown excellent intrarater and good-to-excellent interrater reliability in individuals with acute neck pain.(74) This study reported that the MDC for PPT over the cervical spine and tibialis anterior muscle in patients with acute neck pain was 47.2 and 97.9 kPa, respectively.(74) To determine changes in widespread pressure pain sensitivity, PPTs will be assessed bilaterally over the mid-point trapezius (pars descendens), second metacarpal, and tibialis anterior muscle.
o) Using a pinprick 256 mN wind up ratio will be calculated bilaterally over the mid-point trapezius (pars descendens) and tibiales anterior muscle.(75)

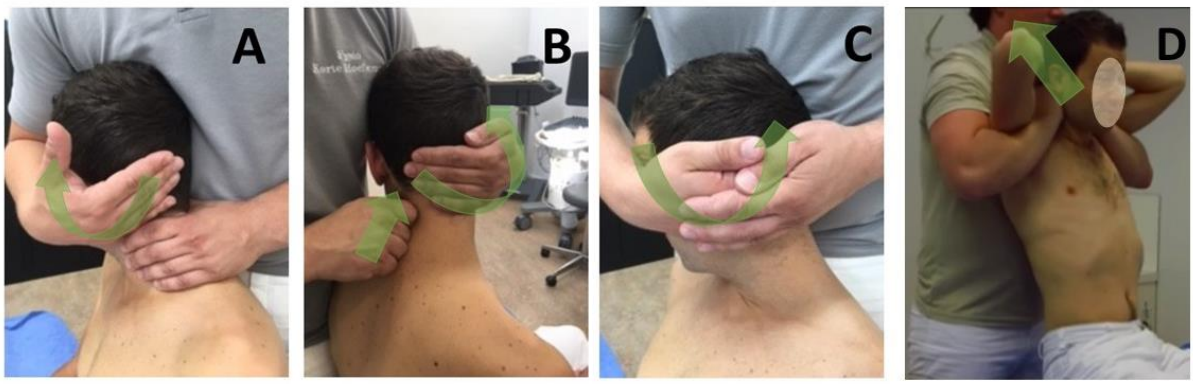
Abbreviations: T0: baseline; T1: immediately following the intervention; T2: two-hours following the intervention; T3: two-days following the intervention

Table 3: Potential confounding variables that will be assessed

Potential confounding variables	
Co-morbidities	Number of co-morbidities
Alcohol use	- Non-drinker - Moderate drinker (women: 1-14 glasses/week) (men: 1-21 glasses/week) - Heavy drinker (women: >14 glasses/week) (men: >21 glasses/week)
Smoking	- Never smoked - Former smoker - Current smoker
Body Mass Index	BMI calculated by dividing body weight (kg) by height (m^2)
Medication use	Type and number of medications used
Drugs use	Recreational drugs use - Yes - No
Visceral Adipose Tissue(76, 77)	Linear distance between abdominal peritoneum and ventral aspect of vertebrae will be assessed using ultrasonography
Physical activity	International Physical Activity Questionnaire, expressed in 1000 metabolic equivalent minutes per week (Dutch version)
Menstrual cycle(78)	Regular menstrual cycle (yes/no), whether women are in the luteal or follicular stage (yes/no), menopause (yes/no) and post menopause (yes/no)
Season(79)	Timing of experiment (summer, autumn, spring or winter)
Age	Age in years
Psychological status(71)	Mental health inventory-5
Intervention expectations(41)	The extent to which they agree (using a four-point Likert scale) with four statements (Appendix A)

Abbreviation: BMI: Body Mass Index


People with persistent non-specific neck pain are recruited from GP practices, primary care physiotherapy practices, and secondary care hospitals
Potential participants are assessed for eligibility and study information will be provided
Exclusion
- Not meeting selection criteria
- Decline to participated
- Other reasons
Written informed consent is obtained
Baseline assessment:
Self-reported questionnaires, physical tests, venipuncture
Randomised and concealed intervention allocation
3:1 (Experimental group:Control group)
Experimental intervention
Control intervention
Immediately following the intervention:
VAS, physical tests, self-reported questionnaires, venipuncture, adverse events
Two-hours following the intervention:
GPE, VAS, physical tests, venipuncture, adverse events
Two-days following the intervention:
GPE, VAS, adverse events



Appendix A: Four statements regarding the intervention expectation of the participants (modified from(41))

I believe this intervention will allow me to get better quicker.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree
I believe this intervention will decrease my neck pain.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree
I believe this intervention will make me more able to do the things I want to do.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree
This seems like a logical way to treat neck pain.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree

TIDieR Template for Intervention Description and Replication

The TIDieR (Template for Intervention Description and Replication) Checklist*:

Information to include when describing an intervention and the location of the information

Item number	Item	Where located **	
		Primary paper (page or appendix number)	Other † (details)
	BRIEF NAME		
1.	Provide the name or a phrase that describes the intervention.	P.4 & P.6	
	WHY		
2.	Describe any rationale, theory, or goal of the elements essential to the intervention.	P.4	
	WHAT		
3.	Materials: Describe any physical or informational materials used in the intervention, including those provided to participants or used in intervention delivery or in training of intervention providers. Provide information on where the materials can be accessed (e.g. online appendix, URL).	P.6, Figure 2 &3	
4.	Procedures: Describe each of the procedures, activities, and/or processes used in the intervention, including any enabling or support activities.	P.4-6	
	WHO PROVIDED		
5.	For each category of intervention provider (e.g. psychologist, nursing assistant), describe their expertise, background and any specific training given.	P.4	
	HOW		
6.	Describe the modes of delivery (e.g. face-to-face or by some other mechanism, such as internet or telephone) of the intervention and whether it was provided individually or in a group.	P.4-6	
	WHERE		
7.	Describe the type(s) of location(s) where the intervention occurred, including any necessary infrastructure or relevant features.	P.3	

	WHEN and HOW MUCH		
8.	Describe the number of times the intervention was delivered and over what period of time including the number of sessions, their schedule, and their duration, intensity or dose.	P2, P4-6	
	TAILORING		
9.	If the intervention was planned to be personalised, titrated or adapted, then describe what, why, when, and how.	P4-5	
	MODIFICATIONS		
10.‡	If the intervention was modified during the course of the study, describe the changes (what, why, when, and how).	N.A.	
	HOW WELL		
11.	Planned: If intervention adherence or fidelity was assessed, describe how and by whom, and if any strategies were used to maintain or improve fidelity, describe them.	N.A.	
12.‡	Actual: If intervention adherence or fidelity was assessed, describe the extent to which the intervention was delivered as planned.	N.A.	

** **Authors** - use N/A if an item is not applicable for the intervention being described. **Reviewers** – use '?' if information about the element is not reported/not sufficiently reported.

† If the information is not provided in the primary paper, give details of where this information is available. This may include locations such as a published protocol or other published papers (provide citation details) or a website (provide the URL).

‡ If completing the TIDieR checklist for a protocol, these items are not relevant to the protocol and cannot be described until the study is complete.

* We strongly recommend using this checklist in conjunction with the TIDieR guide (see *BMJ* 2014;348:g1687) which contains an explanation and elaboration for each item.

* The focus of TIDieR is on reporting details of the intervention elements (and where relevant, comparison elements) of a study. Other elements and methodological features of studies are covered by other reporting statements and checklists and have not been duplicated as part of the TIDieR checklist. When a **randomised trial** is being reported, the TIDieR checklist should be used in conjunction with the CONSORT statement (see www.consort-statement.org) as an extension of **Item 5 of the CONSORT 2010 Statement.** When a **clinical trial protocol** is being reported, the TIDieR checklist should be used in conjunction with the SPIRIT statement as an extension of **Item 11 of the SPIRIT 2013 Statement** (see www.spirit-statement.org). For alternate study designs, TIDieR can be used in conjunction with the appropriate checklist for that study design (see www.equator-network.org).

BMJ Open

Neuroimmune responses following joint mobilisation and manipulation in people with persistent neck pain: A protocol for a randomised placebo-controlled trial

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Neuroimmune responses following joint mobilisation and manipulation in people with persistent neck pain: A protocol for a randomised placebo-controlled trial

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ABSTRACT

***Introduction**:* Joint mobilisation and manipulation often results in immediate pain relief in people with neck pain. However, the biological mechanisms behind pain relief are largely unknown. There is preliminary evidence that joint mobilisation and manipulation lessens the upregulated neuroimmune responses in people with persistent neck pain.

***Methods and analysis**:* This study protocol describes a randomised placebo-controlled trial to investigate whether joint mobilisation and manipulation influence neuroimmune responses in people with persistent neck pain. People with persistent neck pain (N=100) will be allocated, in a randomised and concealed manner, to the experimental or control group (ratio 3:1). Short-term (i.e., baseline, immediately after and two-hours after the intervention) neuroimmune responses will be assessed, such as inflammatory marker concentration following *in-vitro* stimulation of whole blood cells, systemic inflammatory marker concentrations directly from blood samples, phenotypic analysis of peripheral blood mononuclear cells, and serum cortisol. Participants assigned to the experimental group (N=75) will receive cervical mobilisations targeting the painful and/or restricted cervical segments and a distraction manipulation of the cervico-thoracic junction. Participants assigned to the control group (N=25) will receive a placebo mobilisation and placebo manipulation. Using linear mixed models, the short-term neuroimmune responses will be compared 1) between people in the experimental and control group, 2) within the experimental group, between people who experience a good outcome and those with a poor outcome. Furthermore, the association between the short-term neuroimmune responses and pain relief following joint mobilisation and manipulation will be tested in the experimental group.

***Ethics and dissemination**:* This trial is approved by the Medical Ethics Committee of Amsterdam University Medical Centre, location VUmc (Approval number: 2018.181).

***Trial registration number and status**:* The study protocol is registered at trialregister.nl with study ID: NL6575 registered on 18-01-2018; Recruitment commenced 26 February 2019. All data are anticipated to be collected by January 2022, when data analysis and interpretation are anticipated to commence.

Strengths and limitations of this study

- This study provides insight in the interplay between joint mobilisation and manipulation, neuroimmune responses, and pain relief in people with persistent neck pain.
- By adding a placebo-control group, possible working mechanisms of joint mobilisation and manipulation on neuroimmune responses may be revealed.
- The interventions will be delivered by two musculoskeletal physiotherapists, which may limit the generalisability.
- Due to the small control group, it is not feasible to divide the control participants according to outcome.
- Inflammatory indices will be calculated that combine overall inflammatory, pro-inflammatory, anti-inflammatory and ratio pro/anti-inflammatory markers.

1. INTRODUCTION

The disruption of the bidirectional communication pathways between the central nervous system and the immune system may play an important role in persistent pain.(1) Over the last two decades, it has become apparent that neuroimmune crosstalk is present in musculoskeletal pain, and may play a mediating role in the transition from acute to persistent pain.(1) For people with persistent neck pain, aberrant neuroimmune responses may be present, such as systemically elevated levels of inflammatory markers.(2, 3) These increased neuroimmune responses may be relevant to understand and manage persistent spinal pain.(3) A growing body of literature suggests that these neuroimmune responses are associated with pain intensity,(4-6) disability(7) and recovery,(8) and can be influenced by musculoskeletal physiotherapy, such as joint mobilisation and manipulation,(9-11) nerve mobilisation(12, 13) and exercise.(14-16)

Several meta-analyses indicate that musculoskeletal physiotherapy for people with spinal pain may provide immediately pain relief and improvements in functional activities compared to no treatment, placebo or other treatments.(17-19) Nevertheless, unravelling the mechanism of how joint mobilisation and manipulation results in pain relief remains an area for further investigation.(20, 21) There are various explanations of how joint mobilisation and manipulation might cause pain relief, including neurophysiological,(22, 23) neuromuscular,(20) neuroimmune(24, 25) and non-specific responses.(26)

Recent studies suggest a possible neuroimmune-mediated mechanism of pain relief following joint mobilisation and manipulation.(9-11) For example, a reduction in systemic inflammatory marker concentration directly from blood samples(9, 11) and a reduction in inflammatory marker concentration following *in-vitro* stimulation of whole blood cells(10, 27) were found immediately following the intervention. These studies have however

important methodological limitations, such as inclusion of healthy participants,(27) modest sample sizes,(9, 10) a narrow selection of inflammatory markers,(9-11) lack of correction for potential confounding variables,(9, 10, 28) and lack of a placebo-control group.(9, 10) Therefore, we will conduct an adequately powered, placebo-controlled randomised clinical trial in people with persistent neck pain, which will evaluate a broad range of inflammatory markers. The purpose of this paper is to describe the study protocol to investigate the short-term effects of joint mobilisation and manipulation on neuroimmune responses in people with persistent neck pain.

2. METHODS

This manuscript followed the guidelines for clinical trial protocols (SPIRIT statement),(29) for reporting randomised trials (CONSORT statement),(30) and for intervention description and replication (TIDieR checklist).(31)

Aim

The overall aim of this clinical trial is to gain insights in the relation between short-term neuroimmune responses following joint mobilisation and manipulation and pain relief in people with persistent neck pain. The specific aims are: 1) to compare the short-term neuroimmune responses between the experimental and control group; 2) to compare the short-term neuroimmune responses of those in the experimental group with a good outcome (i.e., immediately pain relief) with those in the experimental group with a poor outcome; and 3) to assess the association between short-term neuroimmune responses and pain relief in the experimental group.

Study design and setting

The study is a placebo-controlled randomised trial with follow-up at three time points: baseline, immediately, and two-hours and two-days following the intervention (Fig. 1). Participants will be recruited from GP clinics, primary care physiotherapy practices and outpatient services (neurology and orthopaedic departments) at secondary care hospitals. Data are anticipated to be collected between February 2019 and January 2022, when data analysis and interpretation are anticipated to commence.

Selection criteria

Individuals meeting the following inclusion criteria are eligible to participate: age: 18-65 years; non-specific neck pain for at least six weeks(32) with a minimum pain intensity of 40/100 on a visual analogue scale (VAS), and a sufficient speaking and reading level of the Dutch language to complete the study. Exclusion criteria are contra-indications for cervical mobilisation or cervico-thoracic manipulation,(33, 34) pregnancy or less than 9 months postpartum, contra-indications for venipuncture (e.g., phlebitis), treatment for the current neck pain episode during the preceding two weeks, taken corticosteroids or cytokine modulatory medication (e.g., methotrexate, infliximab) in the preceding 6 weeks, use of botulinum toxin (Botox) injection during the preceding 3 months, non-steroid anti-inflammatory drug medication within the past 7 days (e.g., diclofenac, ibuprofen, naproxen), long-distance flight within the past 7 days, ongoing shift work, having a known comorbid condition with immune/endocrine malfunction (e.g., ankylosing spondylitis, Crohn's disease, sarcoidosis, Cushing syndrome, cancer, diabetes), medical red flags suggestive of serious pathology,(35, 36) and a diagnosed psychological condition (e.g., clinical depression).

Consecutive participants who meet all selection criteria and are willing to participate will be admitted to the study. All participants will provide written informed consent prior to participation. Initial screening for eligibility will be conducted via telephone calls.

Randomisation, concealed allocation and blinding

Block randomisation will be used to allocate participants to the experimental or control group with an allocation ratio of 3:1 (experimental : control). A computer random number generator will create block sizes of 4 and 8 participants. To conceal the allocation sequence, an independent person not involved in the study will assign eligible people to the groups on the day the participant will enrol in the study. Blood samples will be coded to blind the research assistant and laboratory investigators to the study groups. The participant, research assistant and the investigator who includes the participants will be blinded for group assignment. The treating clinicians, research assistant and laboratory investigators will be unaware whether participants experienced a good outcome or not. All laboratory and data analyses will be performed by blinded investigators.

Interventions

Experimental intervention

Spinal mobilisation will consist of low-velocity, low-amplitude mobilisations at the painful cervical segmental levels (Fig. 2 – Panels A-C); spinal manipulation will consist of a high-velocity, low-amplitude distraction manipulation at the cervico-thoracic junction (Fig. 2 – Panel D).(37) These techniques aim to restore motion and reduce pain. They are commonly used and are conform to the Dutch guidelines for musculoskeletal physiotherapy for treating neck pain.(35) All interventions will be performed by two musculoskeletal physiotherapists with more than 5 years of relevant clinical experience.

Cervical mobilisation

Painful and restricted cervical segments will be identified by passive side-bending of the neck targeting each segmental level separately.(38) Reproduction of the participant's pain will be considered to identify the involved level(s). The inter-tester reliability for these tests is fair to substantial.(38, 39)

Depending on the identified painful or restricted spinal levels, the treating clinician may select from different mobilisation techniques: mobilisation targeting the atlanto-axial segment (Fig. 2 – Panel A); segmental zygapophyseal joint mobilisation (C2 to C7) (Fig. 2 – Panel B) and occipital-atlanto-axial joint mobilisation (Fig. 2 – Panel C). Three series of oscillations (~1Hz) will be applied for 30 seconds; with 30 seconds rest in between the series.

Cervico-thoracic junction distraction manipulation

Irrespective of the level of their neck pain, all participants will receive a distraction manipulation of the cervico-thoracic junction (Fig. 2– Panel D).(40) If there is no audible cavitation sound during the first attempt, the manipulation will be repeated once.

Control (placebo) intervention

The control group will receive a placebo mobilisation and placebo manipulation. Procedures, including the instructions, will be identical as for the experimental intervention, except that the clinician will only apply hand contact and no pressure or movement will occur. Participants will be informed that an audible popping sound may or may not occur, and that this sound is not necessary to restore motion and reduce pain.

The credibility of a control intervention can interact with participant expectations in complex ways.(41) To account for differences in intervention expectations, participants will

indicate the extent to which they agree (using a four-point Likert scale) with four statements regarding their intervention expectations (Table 1). These statements will be presented before the delivery of the experimental and control intervention.(42)

Based on the short-term changes in pain intensity score (i.e., immediately and two-hours following the intervention), participants in the experimental group will be categorised into those with a good outcome (≥50% improvement in pain intensity at both time points), a poor outcome (≤20% improvement in pain intensity score at both time points) or an unclear outcome (not fitting the criteria for a good or poor outcome).(43) Based on these cut-off scores, we anticipate to have a minimum of 25 participants in both the good outcome and poor outcome group. If our *a-priori* determined minimum of 25 participants in either group is not achieved, the good outcome group and the poor outcome group will be supplemented with respectively the best responders and poorest responders from the uncertain outcome group in order to obtain 25 participants in both groups.

Outcomes

A broad range of neuroimmune responses will be monitored: a) inflammatory marker concentration following *in-vitro* stimulation of whole blood cells, b) systemic inflammatory marker concentrations directly from blood samples, c) phenotypic analysis of peripheral blood mononuclear cells and d) *ex-vivo* serum cortisol (Table 1). To create an inflammatory profile,(44) a range of pro-inflammatory and anti-inflammatory markers will be used. Ex-vivo serum and supernatants after stimulation will be stored at minus 80°C and will be analysed upon completion of data collection. The laboratory methodology and sample handling prior to stimulation will be tightly monitored and reported, because inconsistency in interlaboratory methodology and reporting impairs interpretation, comparability and reproducibility.(45)

Primary outcomes

The primary outcomes are the short-term (i.e., immediately and two-hours following the intervention) differences in interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) following *in-vitro* stimulation of whole blood cells. These cytokines will be determined using Meso Scale Discovery (MSD, Maryland United States) at baseline, immediately and two-hours following the intervention. These cytokines are selected because previous research has indicated that those cytokines might play a role in spinal pain.(11, 27, 46-48)

To induce cytokine production, whole blood cultures will be stimulated for 24 hours with lipopolysaccharide (LPS) from Escherichia coli O55:B5 (Sigma-Aldrich Chemie GmbH, Schnelldorg, Germany) at a concentration of 1 nanogram LPS/millilitre whole blood (ng/ml) and 10 microgram LPS/millilitre whole blood (µg/ml) at 37°C in a humidified 5% CO_2 incubator. At baseline (Fig. 1) blood samples for neuroimmune measurements (one sodium heparin vacutainer without gel and one serum vacutainer without gel for each time point) will be drawn between 8:00 and 9:00 AM.(49) The cytokine levels will be determined using a custom-made U-plex MSD and expressed in picogram/millilitre (pg/ml). The entire blood stimulation procedure and MSD will be performed by an experienced laboratory technician at Amsterdam University Medical Centre, location VUmc, Department of Clinical Chemistry, Medical Immunology Laboratory.

Secondary outcomes

Several additional neuroimmune responses will be quantified as secondary outcomes at various time points (Table 1).

The levels of interleukin-1 receptor antagonist (IL-1RA), interleukin-4 (IL-4), interleukin-10 (IL-10), c-c motif chemokine ligand 2 (CCL2), c-c motif chemokine ligand 3

(CCL3) and c-c motif chemokine ligand 4 (CCL4) will be determined following *in-vitro* stimulation of whole blood cells.

Systemic inflammatory markers directly from blood samples (tumor necrosis factor – receptor antagonist II (TNF-RII), IL-1β and IL-1RA) will be measured using multianalyte assay Ella (R&D systems, Minneapolis, United States) and high-sensitive c-reactive protein (hsCRP), using Roche/Hitachi cobas c systems (Indianapolis, United States).

To examine a general change in inflammatory marker production, we will calculate *in-vitro* and *ex-vivo* overall inflammatory, pro-inflammatory, anti-inflammatory and ratio pro/anti-inflammatory indices.(50, 51) The indices will be calculated as the mean value or the Ln-transformed data in case of non-normality and z-score standardised levels (based on the control group or poor outcome group) of the inflammatory markers (Appendix A).

Phenotypic analysis of peripheral blood mononuclear cells will be determined. The absolute number of lymphocyte subsets (NK cells, B-cells, $CD4^+$ and $CD8^+$ T-cells and $CD25^{hi}$ regulatory T-cells), monocytes, as well as activation status of these cells, HLA-DR and TLR-4 expression, will be determined by 10-color flowcytometry (FCM, Gallios Flow Cytometer, Beckman Coulter, Indianapolis, United States; Analyse software: Kaluza). Differences between all groups in serum cortisol concentration will be determined using conventional electrochemiluminescence immunoassay (ECLIA) from Roche (Cobas Cortisol, 2nd generation, Indianapolis, United States) in agreement with the manufacturer's protocol.

Procedures

Once consent is obtained (Appendix B), baseline measurements will be taken (Fig. 1). At baseline, participants will undergo physical tests to determine pain characteristics, physical functioning and body composition (Table 2 & Table 3). After this, participants will complete an electronic survey to collect sociodemographic and clinical information (Table 1) and

intervention expectations (Appendix C). Participants will then undergo one venipuncture from the cubital vein to fill two vacutainers which will be used to quantify the neuroimmune responses (Table 1). Collection of all baseline data will take 30-45 minutes and will take place at the Amsterdam University Medical Centre, location VUmc, or at a participating primary care physiotherapy practice, under the supervision of a research assistant.

Participants will then be randomly allocated to the experimental and control group, and treated accordingly. Immediately and two-hours following the intervention, participants will undergo another venipuncture to fill two vacutainers. Between the immediate and two-hours follow-up measures, questionnaires will be completed to collect psychosocial information such as sleep, disability and kinesiophobia (Table 2).

Immediately and two-hours following the intervention, participants will undergo physical tests (Fig. 1) and will rate their pain intensity on a VAS. Two-hours following the intervention, participant will rate their perceived recovery on a 7-point Global Perceived Effect scale (GPE) (Table 2). Two-days following the intervention, participants will receive an electronic survey regarding potential adverse events, GPE and pain intensity. Figure 1 shows the planned flow of participants through the study.

Sample size

Based on the sample size calculation(52) (longitudinal analysis; three time points (baseline, immediately follow-up, two-hours follow-up) with 80% power to detect a mean difference of 550 (SD 933) for TNF-α levels with a 0.05 two-sided significance level, correlation of 0.6 among repeated measures, ratio between groups of 0.25, a total sample size of 91 is needed.(27) Allowing for a drop-out rate of ~10%, a total sample size of 100 participants is required.

Statistical analyses

Data will be checked for normality by the Kolmogorov-Smirnov test and visual inspection of Q-Q plots, box plots and histograms. In case of no normality of data, the data will be log transformation. Data will be presented as means with standard deviations unless otherwise noted. For the analyses, statistical significance will be set at $p<0.05$. Intention-to-treat analyses using mixed models will be performed to analyse differences between the experimental group and control group. Linear mixed model analyses with fixed factor (time), covariate (group) and interaction (time*group) will be used to detect differences between the groups at the three time points (baseline, immediately follow-up, two-hours follow-up) for TNF-α and IL-β following *in-vitro* stimulation of whole blood cells. A random intercept will be selected to account for the correlated nature of multiple measurements from the same participant. The regression coefficient (B), p-value and confidence intervals (95%CI) will be computed for the crude models, as well as for the adjusted models.(28, 53) Linear regression analysis will be used to test for differences in phenotypic analysis of peripheral blood mononuclear cells and cortisol between the experimental and control group and of those in the experimental group with a good outcome (i.e., immediate pain relief) with those in the experimental group with a poor outcome.

Adverse events

Serious and non-serious adverse events related to the experimental and control intervention, and all other aspects of the study, will be documented. At the three post-intervention time points, potential adverse events will be recorded using an online survey. Adverse events will be followed-up as needed by an independent clinician. Depending on the nature of the event, participants may be referred to a GP or a medical specialist, and additional tests or procedures may be proposed. The experimental intervention has been

shown to be safe(11, 27) and it is considered unlikely that serious adverse events due to the interventions will occur. Therefore, installing a data monitoring safety board was not requested by the Ethics Committee.

Patient and public involvement

A panel of four people with persistent neck pain co-developed and evaluated the study design, research questions, choice of experimental and control intervention, and burden of study participation for the participants. Two of these people and two representatives from the public reviewed the Patient information letter and their feedback was used to improve the letter.

Data management and monitoring

The data will be collected at the Department of Rehabilitation of the Amsterdam University Medical Centre, location VUmc, and/or in physiotherapy practices. The collected data will be securely stored at Vrije Universiteit Amsterdam, Faculty of Behavioural and Movement Sciences. All data are de-identified by using unique participant ID numbers in such a way that the data cannot be traced back to the individual participants without the key. The participants code will exist of a random code of three numbers. The electronically key connecting participant names with codes will be kept in a secure location in the principal investigator's office. The key will be kept for six months after the final publication, and will then be destroyed. Data will be stored in a de-identified manner for fifteen years after the final publication.

Role of funding source

This study is funded by the Dutch Association for Manual Therapy (NVMT, grant ID. Top-down_2018) and by the Faculty of Behavioural and Movement Sciences (grant ID. Lab Fund_2019) of Vrije Universiteit Amsterdam. The MSG Science Network (https://www.msg-sciencenetwerk.nl/) (grant ID. N/A) will support participant recruitment. The funding sources have no role in the study design and will not have any roles in data collection, analysis and interpretation of the data, nor in the reporting of the results.

Ethics and dissemination

The study is registered at trialregister.nl with study identification NL6575. The results of the study will be published in peer-reviewed journals and disseminated at conferences, in newsletters and social media. The trial is approved by the Medical Ethics Committee of Amsterdam University Medical Centre, location VUmc (Approval number: 2018.181). All procedures will be conducted in accordance with the Declaration of Helsinki.(54) Amendment to this protocol will be submitted for approval to the Medical Ethical Committee and deviations from the protocol will be reported to the trial registration.

3. DISCUSSION

There is considerable debate in the literature regarding the possibility of meaningful neuroimmune-mediated pain relief following joint mobilisation and manipulation.(2, 7, 55, 56) We described a protocol for a randomised placebo-controlled study that will assess potential neuroimmune-mediated pain relief following joint mobilisation and manipulation in people with persistent neck pain. The aim of this study is to gain insights in the relation

between changes in neuroimmune responses and pain relief, rather than in the clinical efficacy or effectiveness of joint mobilisation and manipulation for people with persistent neck pain.

Recent data suggest that the production of pro-inflammatory cytokines is higher and production of anti-inflammatory cytokines is lower in patients with persistent-pain compared to healthy people following *in-vitro* stimulation of whole blood cells.(44) Additionally, a specific, coordinated inflammatory processes may be important for patient recovery.(3) Contrary to the other studies we are aware of that measured neuroimmune responses following joint mobilisation and manipulation,(7, 44, 57) we will assess a comprehensive range of inflammatory markers. Our approach to measure pro-inflammatory cytokines and their antagonists provides insight into the activation of immunocompetent cells.(58)

We believe the design of our study allows to assess the specific effects of joint mobilisation and manipulation on neuroimmune responses. For instance, rather than comparing the joint mobilisation and manipulation with a wait-and-see approach, we will compare responses with a placebo-control intervention that resembles joint mobilisation and manipulation. Additionally, the verbal instructions between the experimental and control groups will be comparable and standardised, which reduces differences in intervention efficacy due to non-specific intervention effects.(59) Differences in verbal instructions have been shown to be associated with differences in endocrine responses following joint manipulation in people with neck pain.(60) Finally, we will record the participant's intervention expectations and beliefs regarding joint mobilisation and manipulation as a treatment method to alleviate neck pain.(42)

Previous research revealed a non-linearity of the VAS to measure pain intensity, that responsiveness varies along the spectrum of pain intensity and the importance of taking baseline pain into account when evaluating change scores.(43, 61, 62) Consequently, categorising good, unclear and poor outcome using raw data, or change scores in general, are

invalid as these will either underestimate or overestimate true change.(62) To overcome this problem, we follow the initiative on methods, measurement and pain assessment in clinical trials (IMMPACT) recommendation to identify those with a good, poor outcome, or unclear outcome.(43)

Besides the strengths, the proposed study has some potential limitations. First, we assume a linear association between neuroimmune responses and musculoskeletal pain. A linear association between neuroimmune responses and musculoskeletal pain is a prerequisite for the justification of the statistics proposed in this protocol. However, one study suggests that an initial threshold of neuroimmune responses might be required, which would suggest a non-linear relationship between neuroimmune responses and musculoskeletal pain.(63) In that study, elevated IL-6 levels were only present in the group of people with pain > 40/100 VAS compared to control.(63) Therefore, a minimal pain intensity of 40/100 on the VAS will be a prerequisite for participating in this study.

Another limitation is that only a single session of joint mobilisation and manipulation will be provided together with a short follow-up. While a single session of joint mobilisation and manipulation may induce a pain-relieving effect,(17) the clinical relevance of immediately pain relief is unclear. Nonetheless, our aim is not to examine the efficacy of joint mobilisation and manipulation but rather to understand the biological mechanisms behind pain relief following joint mobilisation and manipulation. In studying the mechanism of action, a short follow-up has the advantage that potential confounding variables can be controlled, such as food intake, stress, physical exercise and health status.

Contributorship statement

All authors contributed to the design of this protocol. ILS, GGM and MWC initiated the protocol. The protocol was drafted by ILS, GGM, HJB and MWC. Statistical advice was provided by GGM and MWC. ILS, GGM and MWC were responsible for ethical board approval. ILS was responsible for drafting the manuscript. All authors contributed to the manuscript and read and approved the final manuscript.

Competing interests

The authors have no known conflict of interest and have no commercial interest in this study.

Data sharing statement

Individual deidentified participant data that underlie the results will be shared. Investigators whose proposed use of the data had been approved by an independent review committee identified for this purpose can access the data for individual participant data meta-analysis. Data will be available beginning 9 months and ending 36 months following the result article publication. Proposals may be submitted up to 36 months following article publication. After 36 months the data will be available in our University's data warehouse but without investigator support other than deposited metadata. Information regarding submitting proposals and accessing data may be found at https://research.vu.nl.

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Figure 1: Anticipated flow of the study

Abbreviations: GPE: global perceived effect; VAS: visual analogue scale

Figure 2. Spinal mobilisation and manipulation techniques.

Depending on the identified painful segmental levels, the clinician can select from different cervical mobilisation techniques (A-C); For techniques A-C, the participant will be seated on a chair, leaning against the upper leg or shoulder of the clinician. **Panel A***:* Mobilisation targeting the atlanto-axial joints. The cervical segments below the second cervical vertebrae are submaximal rotated and lateroflexed. With the clinician's hypothenar region of the hand over the structures overlying the arcus of the first vertebrae, the clinician moved the head further in rotation.(40) **Panel B:** Segmental zygapophyseal joint mobilisation (C2 to C7; the image shows the technique for C3-C4). First, the occipital-atlanto-axial joint is maximally rotated in the direction of the facet joint being mobilised. Subsequently, the head is moved to extension, ipsilateral lateroflexion and rotation until pressure from the thumb is felt. This technique is repeated on the lower level until the painful cervical segment is reached (C3-C4). Next, on the painful cervical segment, pressure will be given in a cranio-ventral direction. (40) **Panel C:** Mobilisation technique targeting the occipital-atlanto-axial joints. The clinician's hypothenar region is placed against the mastoid process. C2 to C7 are submaximally locked in flexion, rotation and lateroflexion. The head is then moved in a medio-caudal direction.(40) **Panel D:** Spinal manipulation technique targeting the cervico-thoracic junction. The participant will be seated on a treatment table. The height of the table will be adjusted to the level of the clinician's abdomen. The participant's hands will be placed on the back of their head (with one hand placed over the other hand, rather than with interlocking fingers), and with the shoulders slightly retracted. The clinician's hands will be placed over the hands of the participant, with the clinician's forearms ventral to the shoulder of the participant. Then, a high-velocity, low-amplitude movement will be applied in a dorsal-cranial direction.(40) Green arrows represent the direction of the mobilisation (Panel A-C) or manipulation (Panel D).

Table 1: Overview of the neuroimmune responses

Domain	Neuroimmune parameters	Timing of measurements			
		T0	T1	T2	T3
Systemic inflammatory marker directly from blood samples[a]	TNF-α, TNF-RII, IL-1β, IL-1RA, hsCRP[b]	√	√	√	-
Inflammatory marker concentration after *in-vitro* stimulation of whole blood cells[c]	TNF-α, IL-1β, IL-1RA, IL-4, IL-10, CCL2, CCL3, CCL4	√	√	√	-
Ex-vivo serum cortisol[d]	Cortisol	√	√	-	-
Phenotypic analysis of peripheral blood mononuclear cells[e]	$CD45^+$, $CD3^+$, $CD4^+$, $CD25^{hi}$,$CD8^+$,$CD56^+$, $CD19^+$, $CD14^+$, HLA-DR, TLR-4	√	-	√	-

a) Measured using multianalyte assay Ella (R&D systems, Minneapolis, United States)
b) Cardiac C-Reactive Protein (Latex) High Sensitive using Roche/Hitachi cobas c systems.
c) Stimulated for 24 hours at 37°C, in a humidified 5% CO_2 incubator, with lipopolysaccharide (LPS) from *Escherichia coli O55:B5* at a concentration of 1ng/ml and 10µg/ml. Determined using a custom-made U-plex (MSD, Maryland, United States)
d) Using conventional electrochemiluminescence immunoassay (ECLIA), Roche (Cobas Cortisol, 2nd generation).
e) Determined by 10-color flowcytometry (FCM): CD45+ = General Leukocyte marker; CD3+ = T-cell marker; CD3+CD4+ = CD4+ T-helper marker; CD3+CD4+CD25hi = T-regulator cell marker; CD3+CD8+ = Cytotoxic T-cell marker; CD3-CD56+ = Natural Killer cell marker; CD19+ = B-cell marker; CD14+ = monocyte marker; HLA-DR = activation marker for T-cells and monocytes; TLR-4 = Toll-like receptor 4 marker.

Abbreviations: T0: baseline; T1: immediately following the intervention; T2: two-hours following the intervention; T3: two-days following the intervention; TNF-α: Tumor Necrosis Factor-α; TNF-RII: Tumor Necrosis Factor Receptor Antagonist 2; IL-1β: Interleukin-1β; IL-1RA: Interleukin-1 receptor antagonist; hsCRP: High sensitive C-Reactive Protein; IL-4: Interleukin-4; IL-10: Interleukin-10; CCL2: c-c-motif chemokine ligand 2; CCL3: c-c-motif chemokine ligand 3; CCL4: c-c-motif chemokine ligand 4; CD: Cluster of Differentiation

Table 2: Self-reported questionnaires and physical tests

Domain	Self-reported questionnaires	Timing of measurements			
		T0	T1	T2	T3
Disability	Neck Disability Index (NDI)[a]	-	√	-	-
Perceived effect	Global Perceived Effect (GPE)[b]	-	-	√	√
Fear of movement	Tampa Scale of Kinesiophobia[c]	-	√	-	-
Type of pain	PAIN Detect Questionnaire (PDQ)[d]	-	√	-	-
Type of pain	Central Sensitisation Inventory (CSI)[e]	-	√	-	-
Depression, Anxiety, Stress	Depression Anxiety Stress Scale (DASS21)[f]	-	√	-	-
Physical activity	International Physical Activity Questionnaire (IPAQ)[g]	-	√	-	-
Catastrophising	Pain Catastrophising Scale (PCS)[h]	-	√	-	-
Sleep Quality	Pittsburgh Sleep Quality Index (PSQI)[i]	-	√	-	-
Pain Intensity	Visual Analogue Scale (VAS)[j]	√	√	√	√
Mental health	Mental health inventory (MHI-5)[k]	√	-	-	-
Domain	**Physical tests**	**Timing of measurement**			
		T0	**T1**	**T2**	**T3**
Range of motion	Cervical Range of Motion (CROM)[l]	√	√	√	-
Pain intensity	CROM-VAS test[m]	-	√	√	-
Quantitative sensory testing	Pressure Pain Threshold (PPT)[n]	√	√	√	-
Quantitative sensory testing	Wind-up ratio[o]	√	√	√	-

a) The Dutch version of the NDI is a valid and responsive measure of disability.(64)
b) The GPE is a validated and reliable tool to assess health transitions in patients with musculoskeletal disorders.(65)
c) Preferred self-administrated questionnaire to asses fear of movement in musculoskeletal pain.(66)
d) Persistent pain will be categorised in two-mechanism based groups: nociceptive and neuropathic pain using the PDQ. The PD-Q is a reliable screening tool with high specificity.(67)
e) The Dutch Central Sensitization Inventory (CSI) has good internal consistency, good discriminative power and excellent test-retest reliability. A cut-off score of 40/100 provides a sensitivity of 81% and specificity of 75%.(68)

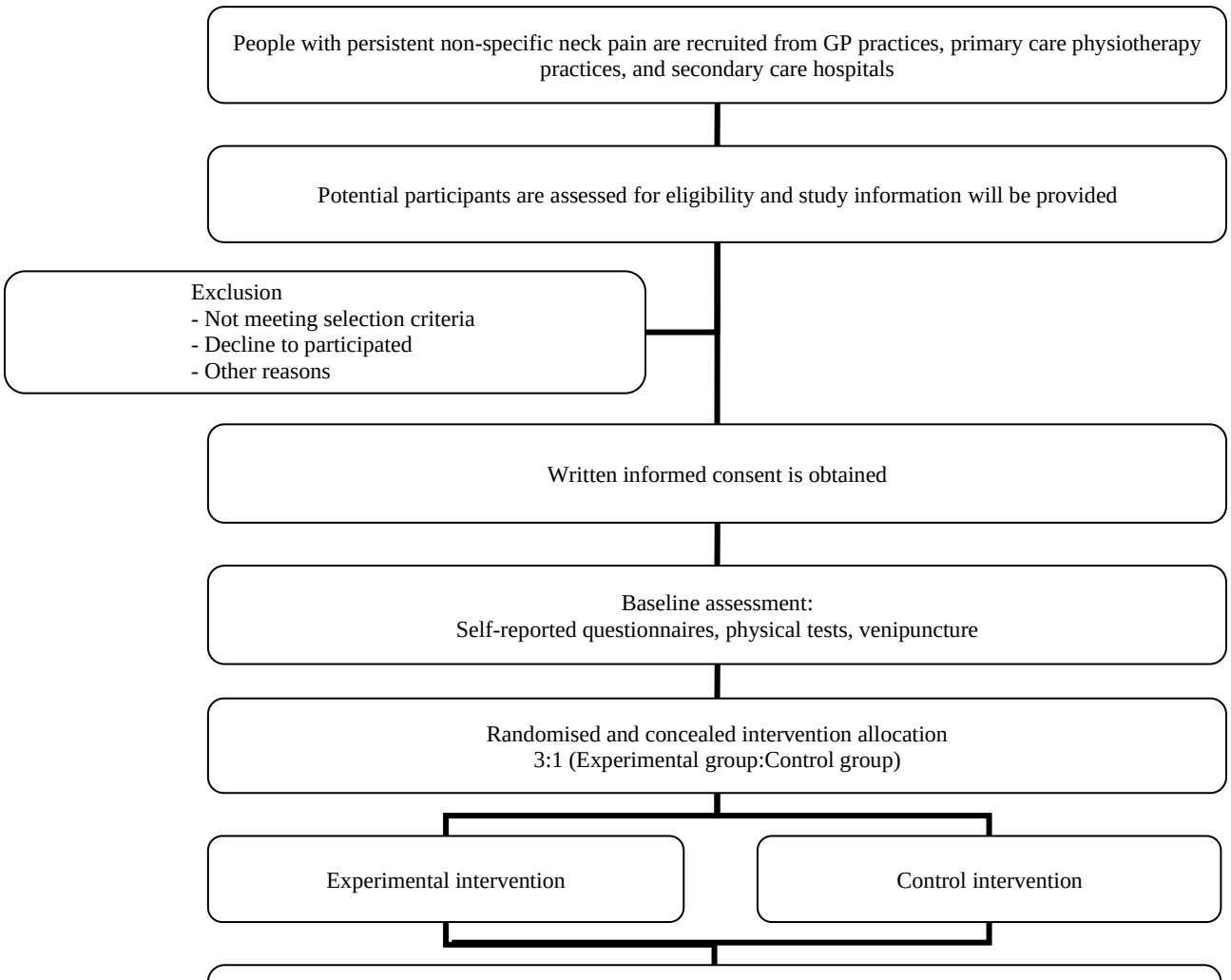
f) Preferred self-administrated questionnaire to assess depression, anxiety and stress in musculoskeletal pain.(66, 69)
g) Expressed in 1000 metabolic equivalent minutes per week (Dutch-language version).(70) The IPAQ has good reliability (intraclass correlation coefficient [ICC] = 0.70-0.96) and moderate validity (r = 0.36-0.49) of the IPAQ compared with an accelerometer.(71)
h) Preferred self-administrated questionnaire to assess pain catastrophising in musculoskeletal pain.(66)
i) Score above 5 yield a sensitivity of 89.6% and specificity of 86.5% in distinguishing good and poor sleepers.(72)
j) The reliability and validity of the VAS as a measure of pain for neck pain patients is good.(73)
k) General psychological status will be assessed using the MHI-5.(74) A higher score indicates better mental health. Cronbach's alpha for the MHI-5 scale is 0.85.(75)
l) The CROM is a clinically reliable tool to measure active cervical range of motion people with neck pain and healthy participants.(76)
m) This novel test consists of two parts. In Part 1, the participant is asked to perform maximal active right and left cervical rotation and the degrees of rotation are reordered using the CROM device. In this position, the pain intensity is measured with the VAS following intervention. After the intervention, Part 2 of the test is performed . The participant is again asked to actively rotate (left and right) to the same position as in Part 1 and the pain intensity is recorded. The difference on VAS scores is the outcome of the CROM-VAS test.
n) Pressure algometry over the cervical spine has shown excellent intrarater and good-to-excellent interrater reliability in individuals with acute neck pain.(77) This study reported that the MDC for PPT over the cervical spine and tibialis anterior muscle in patients with acute neck pain was 47.2 and 97.9 kPa, respectively.(77) To determine changes in widespread pressure pain sensitivity, PPTs will be assessed bilaterally over the mid-point trapezius (pars descendens), second metacarpal, and tibialis anterior muscle.
o) Using a pinprick 256 mN wind up ratio will be calculated bilaterally over the mid-point trapezius (pars descendens) and tibiales anterior muscle.(78)

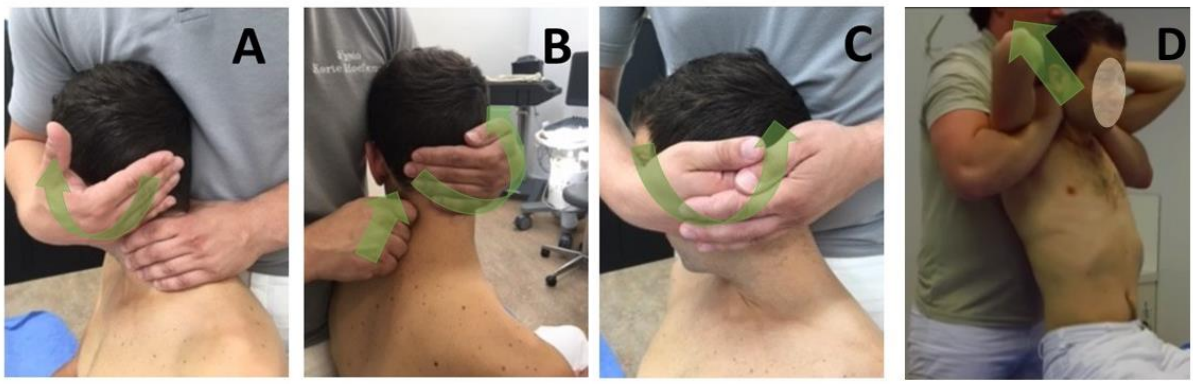
Abbreviations: T0: baseline; T1: immediately following the intervention; T2: two-hours following the intervention; T3: two-days following the intervention

Table 3: Potential confounding variables that will be assessed

Potential confounding variables	
Co-morbidities	Number of co-morbidities
Alcohol use	- Non-drinker - Moderate drinker (women: 1-14 glasses/week) (men: 1-21 glasses/week) - Heavy drinker (women: >14 glasses/week) (men: >21 glasses/week)
Smoking	- Never smoked - Former smoker - Current smoker
Body Mass Index	BMI calculated by dividing body weight (kg) by height (m^2)
Medication use	Type and number of medications used
Drugs use	Recreational drugs use - Yes - No
Visceral Adipose Tissue(79, 80)	Linear distance between abdominal peritoneum and ventral aspect of vertebrae will be assessed using ultrasonography
Physical activity	International Physical Activity Questionnaire, expressed in 1000 metabolic equivalent minutes per week (Dutch version)
Menstrual cycle(81)	Regular menstrual cycle (yes/no), whether women are in the luteal or follicular stage (yes/no), menopause (yes/no) and post menopause (yes/no)
Season(82)	Timing of experiment (summer, autumn, spring or winter)
Age	Age in years
Psychological status(74)	Mental health inventory-5
Intervention expectations(42)	The extent to which they agree (using a four-point Likert scale) with four statements (Appendix C)

Abbreviation: BMI: Body Mass Index


People with persistent non-specific neck pain are recruited from GP practices, primary care physiotherapy practices, and secondary care hospitals
Potential participants are assessed for eligibility and study information will be provided
Exclusion
- Not meeting selection criteria
- Decline to participated
- Other reasons
Written informed consent is obtained
Baseline assessment:
Self-reported questionnaires, physical tests, venipuncture
Randomised and concealed intervention allocation
3:1 (Experimental group:Control group)
Experimental intervention
Control intervention
Immediately following the intervention:
VAS, physical tests, self-reported questionnaires, venipuncture, adverse events
Two-hours following the intervention:
GPE, VAS, physical tests, venipuncture, adverse events
Two-days following the intervention:
GPE, VAS, adverse events



Appendix A: Inflammatory indices

The inflammatory indices will be calculated as follows: The *ex-vivo* and *in-vitro* inflammatory markers will be z-score standardised, summed and divided by the total number of inflammatory markers used. Z-score standardisation for the experimental intervention and control intervention will be obtained using the T0-mean and T0-standard deviation of the control intervention. Z-score standardisation for those in the experimental group with a good outcome and those in the experimental group with a poor outcome will be obtained using the T0-mean and T0-standard deviation of the poor outcome group. Inflammatory markers will be Ln-transformed in the case of non-normality. The following calculations will be used to determine the separate indices:

$$ex\text{-}vivo \text{ inflammatory index} = \frac{zTNF\alpha + zTNFR2 + zIL1\beta + zIL1RA + zhsCRP}{5}$$

$$ex\text{-}vivo \text{ pro inflammatory index} = \frac{zTNF\alpha + zIL1\beta + zhsCRP}{3}$$

$$ex\text{-}vivo \text{ anti inflammatory index} = \frac{zTNFR2 + zIL1RA}{2}$$

$$ex\text{-}vivo \text{ ratio} \frac{\text{pro}}{\text{anti}} \text{inflammatory index} = \frac{ex - vivo\ pro\ inflammatory\ index}{ex - vivo\ anti\ inflammatory\ index}$$

$$in\text{-}vitro \text{ inflammatory index} = \frac{zTNF\alpha + zIL1\beta + zIL1RA + zIL4 + zIL10 + zCCL2 + zCCL3 + zCCL4 + zhsCRP}{9}$$

$$in\text{-}vitro \text{ pro inflammatory index} = \frac{zTNF\alpha + zIL1\beta + zCCL2 + zCCL3 + zCCL4 + zhsCRP}{6}$$

$$in\text{-}vitro \text{ anti inflammatory index} = \frac{zIL1RA + zIL4 + zIL10}{3}$$

$$in\text{-}vitro \text{ ratio} \frac{\text{pro}}{\text{anti}} \text{inflammatory index} = \frac{in - vitro\ pro\ inflammatory\ index}{in - vitro\ anti\ inflammatory\ index}$$

Appendix B: Patient informed consent form

- *Ik heb de informatiebrief gelezen. Ook kon ik vragen stellen. Mijn vragen zijn voldoende beantwoord. Ik had genoeg tijd om te beslissen of ik meedoe.*
- *Ik weet dat meedoen vrijwillig is. Ook weet ik dat ik op ieder moment kan beslissen om toch niet mee te doen of te stoppen met het onderzoek. Daarvoor hoef ik geen reden te geven.*
- *Ik geef toestemming voor het informeren van mijn huisarts dat ik meedoe aan dit onderzoek.*
- *Ik geef toestemming voor het verzamelen en gebruiken van mijn gegevens en bloedmonsters voor de beantwoording van de onderzoeksvraag in dit onderzoek.*
- *Ik weet dat voor de controle van het onderzoek sommige mensen toegang tot al mijn gegevens kunnen krijgen. Die mensen staan vermeld in deze informatiebrief. Ik geef toestemming voor die inzage door deze personen.*
- *Ik geef toestemming voor het informeren van mijn huisarts en/of behandelend specialist van onverwachte bevindingen die van belang (kunnen) zijn voor mijn gezondheid.*
- *Ik geef toestemming dat mijn huisarts mij mag informeren over onverwachte bevindingen die van belang (kunnen) zijn voor mijn gezondheid.*
- *Ik geef toestemming om mijn gegevens nog 15 jaar na dit onderzoek te bewaren. Mogelijk kan dit later nog voor [ander/meer] onderzoek worden gebruikt.*
- *Ik ben me ervan bewust dat de gegeven interventie geen vervanging biedt voor een volledig manueel therapeutische behandeling*

- *Ik geef* □ ***wel***

  □ ***geen***

  Toestemming om drie extra bloedsamples (3 keer 5 ml) af te nemen en te bewaren (5 jaar) en om dit later nog voor ander/meer onderzoek te gebruiken, zoals in de informatiebrief staat.

- *Ik wil* □ ***wel***

  □ ***niet***

  Geïnformeerd worden over de uitkomsten van het onderzoek. Dit is informatie over het hele onderzoek en niet specifiek op mij toegespitst.

Toestemmingsformulier

Voor- en achternaam: _____

Straatnaam en huisnummer: _____

Postcode en Woonplaats: _____

E-mailadres:

_____

Handtekening: *Datum:* __ / __ / __

In te vullen door de uitvoerende onderzoeker

- *Ik verklaar dat ik deze proefpersoon volledig heb geïnformeerd over het genoemde onderzoek.*
- *Als er tijdens het onderzoek informatie bekend wordt die de toestemming van de proefpersoon zou kunnen beïnvloeden, dan breng ik hem/haar daarvan tijdig op de hoogte.*
- *De proefpersoon krijgt een volledige informatiebrief mee, samen met een kopie van het getekende toestemmingsformulier.*

Naam onderzoeker: *Datum :* __ / __ / __

Handtekening:

Appendix C: Four statements regarding the intervention expectation of the participants (modified from(41))

I believe this intervention will allow me to get better quicker.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree
I believe this intervention will decrease my neck pain.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree
I believe this intervention will make me more able to do the things I want to do.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree
This seems like a logical way to treat neck pain.	☐ Strongly agree ☐ Agree ☐ Disagree ☐ Strongly disagree

TIDieR Template for Intervention Description and Replication

The TIDieR (Template for Intervention Description and Replication) Checklist*:

Information to include when describing an intervention and the location of the information

Item number	Item	Where located **	
		Primary paper (page or appendix number)	Other † (details)
	BRIEF NAME		
1.	Provide the name or a phrase that describes the intervention.	P.4 & P.6	
	WHY		
2.	Describe any rationale, theory, or goal of the elements essential to the intervention.	P.4	
	WHAT		
3.	Materials: Describe any physical or informational materials used in the intervention, including those provided to participants or used in intervention delivery or in training of intervention providers. Provide information on where the materials can be accessed (e.g. online appendix, URL).	P.6, Figure 2 &3	
4.	Procedures: Describe each of the procedures, activities, and/or processes used in the intervention, including any enabling or support activities.	P.4-6	
	WHO PROVIDED		
5.	For each category of intervention provider (e.g. psychologist, nursing assistant), describe their expertise, background and any specific training given.	P.4	
	HOW		
6.	Describe the modes of delivery (e.g. face-to-face or by some other mechanism, such as internet or telephone) of the intervention and whether it was provided individually or in a group.	P.4-6	
	WHERE		
7.	Describe the type(s) of location(s) where the intervention occurred, including any necessary infrastructure or relevant features.	P.3	

	WHEN and HOW MUCH		
8.	Describe the number of times the intervention was delivered and over what period of time including the number of sessions, their schedule, and their duration, intensity or dose.	P2, P4-6	
	TAILORING		
9.	If the intervention was planned to be personalised, titrated or adapted, then describe what, why, when, and how.	P4-5	
	MODIFICATIONS		
10.‡	If the intervention was modified during the course of the study, describe the changes (what, why, when, and how).	N.A.	
	HOW WELL		
11.	Planned: If intervention adherence or fidelity was assessed, describe how and by whom, and if any strategies were used to maintain or improve fidelity, describe them.	N.A.	
12.‡	Actual: If intervention adherence or fidelity was assessed, describe the extent to which the intervention was delivered as planned.	N.A.	

** **Authors** - use N/A if an item is not applicable for the intervention being described. **Reviewers** – use '?' if information about the element is not reported/not sufficiently reported.

† If the information is not provided in the primary paper, give details of where this information is available. This may include locations such as a published protocol or other published papers (provide citation details) or a website (provide the URL).

‡ If completing the TIDieR checklist for a protocol, these items are not relevant to the protocol and cannot be described until the study is complete.

* We strongly recommend using this checklist in conjunction with the TIDieR guide (see *BMJ* 2014;348:g1687) which contains an explanation and elaboration for each item.

* The focus of TIDieR is on reporting details of the intervention elements (and where relevant, comparison elements) of a study. Other elements and methodological features of studies are covered by other reporting statements and checklists and have not been duplicated as part of the TIDieR checklist. When a **randomised trial** is being reported, the TIDieR checklist should be used in conjunction with the CONSORT statement (see www.consort-statement.org) as an extension of **Item 5 of the CONSORT 2010 Statement.** When a **clinical trial protocol** is being reported, the TIDieR checklist should be used in conjunction with the SPIRIT statement as an extension of **Item 11 of the SPIRIT 2013 Statement** (see www.spirit-statement.org). For alternate study designs, TIDieR can be used in conjunction with the appropriate checklist for that study design (see www.equator-network.org).

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	ItemNo	Description	Page / line number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Page 1; lines 1-2
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Page 3; lines 3-6
	2b	All items from the World Health Organization Trial Registration Data Set	
Protocol version	3	Date and version identifier	Page 3; line 4
Funding	4	Sources and types of financial, material, and other support	Page 18; lines10-12
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Page 1; lines 4-17 Page 18; lines 1-4
	5b	Name and contact information for the trial sponsor	Page 1; lines26-35
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	Page 15; lines1-7
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Page 15; lines 10-16

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Page 4-5; lines 21-25; 1-3
	6b	Explanation for choice of comparators	Page 5; line 3
Objectives	7	Specific objectives or hypotheses	Page 5; lines 17-24
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	Page 5; lines 4-5
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Page 6; 2-5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Page 6; lines 10-23
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Pages 7-8; lines 19-25; Lines 2-23
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	n.a.
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	n.a.
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	n.a.

Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Page 10-11; lines 1-24 Lines 1-19
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Page 29; line 2 (Figure 1)
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Page 12; lines 19-24
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Page 6; lines 4-5
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Page 7; lines 6-8
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Page 7; lines 8-10
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Page 7; lines 8-10
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Page 7; lines 11-15

	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	n.a.
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Page 9-10; lines 16-14 Lines 1-18
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	n.a.
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Page 14; lines 13-22
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Page 13; lines 2-17
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Page 13; line 13
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	n.a.
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	Page 14; lines 2-3

	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	n.a.
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	Page 13; lines 20-25
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	n.a.
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Page 15; lines 10-16
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	Page 15; lines 10-16
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Page 7; line 12
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n.a.
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Page 14; lines 13-22
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Page 15; lines 10-16 Page 18; line 7
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Page 18; lines 10-17

Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	Page 13; line 23
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Page 14; line 10
	31b	Authorship eligibility guidelines and any intended use of professional writers	Page 18; lines 2-4
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Page 18; lines 10-17
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	Page 35
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	n.a.

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.