Statistical Analysis Plan
Version 1.0
30/03/2023

A Randomised Controlled Trial to Investigate the Effects of Parental Touch on Relieving Acute Procedural Pain in Neonates

Trial Protocol version: 5.0 (26-01-2023)

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This statistical analysis plan follows the guidelines by Gamble and colleagues (Gamble et al., 2017).

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List of abbreviations

CT	C-Tactile
ECG	Electrocardiography
EEG	Electroencephalography
IRAS	Integrated Research Application System
ISRCTN	International Standard Randomised Controlled Trial Number
IVH	Intraventricular Haemorrhage
PIPP-R	Premature Infant Pain Profile – Revised
SD	Standard Deviation
SSNAP	Supporting the Sick Newborn And their Parents
STAI	State-Trait Anxiety Inventory

1 Section 1: Administrative information

1.1 Trial registration

Short Title: Parental touch trial (*Petal*)
Long Title: A randomised controlled trial to investigate the effects of parental touch on relieving acute procedural pain in neonates
IRAS ref: 291213
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1.2 SAP Version

SAP v2.0 (30-03-2023)

1.3 Protocol version

Trial protocol v5.0 (26-01-2023)

1.4 Roles

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2 Section 2: Introduction

This document describes the statistical analysis plan of the Parental Touch Trial (*Petal*), a randomised controlled trial investigating the effects of parental touch on relieving acute procedural pain in neonates. The protocol for this trial has been published in BMJ Open (Cobo et al., 2022). This statistical analysis plan will be published online before any comparative analyses are performed.

2.1 Background and rationale

2.1.1 Background

Newborn infants undergo painful procedures as part of routine postnatal care. Sick or premature infants experience an average of 10 painful procedures per day as part of life-sustaining treatment (Carbajal et al., 2008). Measuring pain in non-verbal infants is challenging, and few safe and effective analgesics have been tested and approved for use in infants. Non-pharmacological pain-relieving strategies have been introduced over the last few decades including sweet-taste solutions, breastfeeding, and swaddling. Sweet-taste solutions such as sucrose are effective in relieving behavioural responses following minor painful procedures (Stevens et al., 2013). However, this commonly promoted pain-relieving strategy does not reduce noxious input to the brain (Slater et al., 2010), causing concern that it may not mitigate long-term consequences of early life pain, and it may have long-term neurodevelopmental effects with repeated use (Ranger et al., 2019; Schneider et al., 2018; Tremblay et al., 2017). Breastfeeding also reduces behavioural and physiological responses to pain in full-term infants undergoing heel lancing, intramuscular injection, and venepuncture (Benoit et al., 2017). However, this strategy can be challenging for new mothers and is not always practical to implement, for example in premature and critically ill infants, in mothers with transmissible infections, and due to maternal concerns regarding potential negative impact on breastfeeding when it is coupled to a painful procedure (Tansky & Lindberg, 2010). Other comfort measures include swaddling and facilitated tucking of infants, which, although useful, are less effective in reducing pain (Meek & Huertas, 2012). Despite guidelines recommending the use of nonpharmacological interventions for pain relief, uptake of these practices is poor and inconsistent (Courtois et al., 2016; Losacco et al., 2011).

Untreated repetitive pain in neonates can cause short-term physiological instability as well as long-term neurodevelopmental consequences such as reduced growth, altered structural and functional brain development, and reduced school-age academic performance (Walker, 2019). Poor management of neonatal pain also has a negative impact on parents. Mothers of hospitalised infants report feeling emotionally and psychologically traumatised due to having to allow their infants to undergo clinically necessary painful procedures, and due to feelings of helplessness from being unable to protect or comfort their child (Franck et al., 2005; Gale et al., 2004; Kyololo et al., 2019). Actively involving parents in care relieves parental distress (Franck et al., 2012) and increases the likelihood that infants receive treatment for pain (Carbajal et al., 2008; C. Johnston et al., 2011; Kyololo et al., 2019). Furthermore, there is evidence to suggest that parents of healthy infants are increasingly refusing clinically recommended interventions such as vitamin-K intramuscular injections at birth (Nicholls & Southern, 2013) and immunisations (Harrington et al., 2000; Mills et al., 2005; Tickner et al., 2010) due to anxiety about pain. Avoidance of key interventions in early life could have drastic

consequences for child health and this issue must be addressed. Indeed, parental anxiety and attitudes during painful procedures can impact neonatal distress and subsequent pain experience during clinical procedures in later life (Racine et al., 2016). Parental anxiety regarding pain could be alleviated by empowering parents to provide safe and effective pain relief for their child. Parental touch through stroking is an instinctive parental behaviour which may provide a simple parent-led intervention to alleviate procedural pain in infants by activating their innate C-tactile (CT) fibre system to naturally reduce noxious input reaching the brain.

2.1.2 Existing Evidence

2.1.2.1 Pain-relieving tactile interventions

Skin-to-skin contact and kangaroo care are comfort measures consisting of ventral skin contact of the newborn with the caregiver's chest. Skin-to-skin contact has been shown to reduce behavioural and physiological indicators of infant pain to acute noxious procedures including heel lancing (de Sousa Freire et al., 2008; Gray et al., 2000; C. C. Johnston et al., 2003), venepuncture (Akcan et al., 2009), and intramuscular injection (Chermont et al., 2009), and has been recommended for pain relief during blood sampling (Howard et al., 2008). A Cochrane review of 25 studies concluded that skin-to-skin contact is safe and effective in reducing physiological (heart rate) and behavioural (crying time) indicators of pain following clinically required painful procedures (C. Johnston et al., 2017). However, the quality of evidence for an effect on acute pain response was low. Infant massage is another tactile comfort measure in which stroking and passive movements are performed at intervals (T. M. Field et al., 1986). Across a range of painful procedures including heel lancing, venepuncture and tape removal, infant massage has been shown to decrease facial expressions and heart rate responses in both term and premature infants (Abdallah et al., 2013; Bellieni et al., 2007; Chik et al., 2017; Diego et al., 2009; Hathaway et al., 2015; Jain et al., 2006; Zargham-Boroujeni et al., 2017). While many studies report the potential pain-relieving effects of tactile interventions in the context of minor painful procedures, these simple non-pharmacological interventions are scarcely used in maternity and neonatal units (T. Field et al., 2006) and the mechanisms underpinning the effectiveness of these interventions are still being established. Barriers to use likely include environmental issues, negative staff attitudes, lack of help from staff and limited awareness of the potential benefits (Seidman et al., 2015).

2.1.2.2 Stroking and C-tactile (CT)-fibres

Maternal touch behaviours are instinctive, evolutionarily conserved amongst mammals (Hertenstein et al., 2006) and enhance infant growth (T. Field et al., 2011) and development (Vickers et al., 2004). Stroking, by repeatedly applying gentle pressure to the skin, can activate CT fibres, a subclass of slow-conducting unmyelinated sensory neurons, mostly found on hairy skin (Liu et al., 2007; A. Vallbo et al., 1993; A. B. Vallbo et al., 1995). These fibres project to brain regions associated with affective processing such as the insular cortex, prefrontal cortex, superior temporal sulcus, and cingulate cortex (Bennett et al., 2014; Gordon et al., 2013; McGlone et al., 2012; Morrison, Björnsdotter, et al., 2011; Olausson et al., 2002) and are thought to have evolved to promote affiliative behaviours and social touch (McGlone et al., 2014; Morrison, Löken, et al., 2011; Olausson et al., 2010; von Mohr et al., 2017). CT fibres are optimally activated by stroking at a velocity of 3 cm/s (optimal range 1-10 cm/s) (Essick et

al., 2010; Löken et al., 2009; Triscoli et al., 2014). In adults, activation of CT-fibres using gentle brushing or stroking paradigms has been shown to reduce pain. Stroking the skin at a CT-optimal rate prior to an experimental thermal pain stimulus reduces pain ratings compared to CT non-optimal touch (velocity 30 cm/s) (Liljencrantz et al., 2017). Using EEG, a study has demonstrated that CT optimal touch reduces noxious-evoked brain activity arising from laser stimulation, in addition to reducing pain ratings (von Mohr et al., 2018). CT-optimal stimulation therefore could provide a natural and safe pain-relieving intervention in infants.

2.1.3 Clinical Measures of Pain in Infants

Pain assessment in infants primarily relies on measuring changes in infant behaviour in response to noxious events. In premature infants, a common validated clinical pain tool is the Premature Infant Pain Profile (PIPP) and its revised version, the PIPP-R (Stevens et al., 1996; Stevens et al., 2014). As PIPP and PIPP-R are composite multimodal measures, incorporating measures of heart rate, oxygen saturation and facial expression change, they allow for different aspects of the infant pain response to be captured. The score has been widely used as the primary outcome measure for infant pain in many clinical trials (Campbell-Yeo et al., 2012; Simonse et al., 2012; Taddio et al., 2008). If parental touch prior to a heel lance significantly reduces subsequent pain scores, there will be a strong rationale for its use in clinical practice.

2.1.4 Measures of Noxious-evoked Brain Activity

While behavioural measures are presently the most prevalent measures for the assessment of neonatal pain, electrophysiological techniques have more recently been developed to identify patterns of noxious-evoked brain activity. Noxious-evoked patterns of brain activity have been well-characterised following clinical heel lancing with electroencephalography (EEG), (Fabrizi et al., 2011; Hartley et al., 2017; Slater, Worley, et al., 2010; Worley et al., 2012), and a template of noxious-evoked brain activity has been developed and validated (Hartley et al., 2017). Noxious-evoked brain activity is an objective and quantifiable neurophysiological measure, which has been used as the primary outcome measure in a clinical trial published in *The Lancet,* providing evidence to suggest that sucrose may not provide analgesia during clinical heel lancing (Slater et al., 2010). Furthermore, it has been used as co-primary outcome in the Poppi trial, a randomised controlled trial also published in *The Lancet* assessing the analgesic efficacy and safety of morphine for acute procedural pain in infants (Hartley et al., 2018). In this clinical trial, noxious-evoked brain activity following a heel lance will be used to investigate the efficacy of parental touch.

Heel lancing is a clinical procedure frequently performed in neonates to provide blood samples for the monitoring of jaundice, blood sugar, electrolytes and other haematological parameters. Heel lancing is one of the two acute pain models recommended for neonate clinical trials by the Analgesic, Anesthetic, and Addiction Clinical Trial Translations, Innovations, Opportunities, and Networks (ACTTION) Pediatric Pain Research Consortium (Walco et al., 2018).

2.1.5 Benefits and Risks

The benefit of this trial is that we hope to determine whether pre-emptive parental touch prior to a heel lance (through stroking) provides effective pain relief for acute procedural pain in neonates. Effective pain relief could make infants more comfortable during these procedures, and the provision of effective parental-led pain relief may prevent some of short and long-term consequences linked to infant pain.

Parental participation in this trial may also have a positive impact on parental wellbeing. The simple tactile intervention we propose could improve parental wellbeing by empowering them to take an active role in providing comfort and pain relief, reducing their anxiety and stress, increasing confidence (Skene et al., 2012) and addressing commonly experienced feelings of helplessness and lack of involvement (Kyololo et al., 2019). Unlike other non-pharmacological interventions, this strategy could be implemented regardless of feeding status of the infant or availability of a resource like sucrose.

Infant massage, a tactile comfort measure which involves patterns of stroking, has been shown to improve mother-infant bonding and improve postnatal depression (Onozawa et al., 2001), a condition affecting at least one in ten UK mothers in the first year post-delivery (Petersen et al., 2018). Furthermore, maternal stroking of infants has been shown to moderate the behavioural and physiological effects of maternal depression on infants (Sharp et al., 2012). Promoting parental stroking and exploiting this natural tactile behaviour to provide evidence-based pain-relief would be beneficial to both mothers and infants.

Greater parental involvement in comfort care can improve a family's experience of healthcare (Cooper et al., 2007) and strengthen the parent-physician relationship (Skene et al., 2012). Reducing parental anxiety regarding procedural pain could reduce parental refusal of clinically necessary minor painful procedures such as blood tests and vaccinations, providing a considerable public health benefit.

2.1.6 Trial Justification

In the UK, more than 100,000 babies receive neonatal intensive care every year as a result of prematurity or illness (NICE, 2019). All newborns are exposed to clinically necessary painful procedures. Even healthy neonates on postnatal wards can require repeated painful procedures beyond routine Newborn Screening, such as blood tests for glucose monitoring or jaundice, which can be distressing for both neonates and parents. As such, improving the management of pain is recognised as a top neonatal UK research priority (Duley et al., 2014) and a major concern amongst parents and neonatal nurses (Wielenga et al., 2015). This trial investigates a simple, free, low-risk, non-pharmacological pain-relieving intervention, which could be delivered by parents in partnership with physicians. Following evidence of clinical benefit, this intervention could be rolled out almost immediately through standard NHS clinical guideline adoption processes.

We previously conducted a study on the effect of pre-procedural stroking on pain relief in neonates (Gursul et al., 2018), in which we demonstrated that CT-optimal stroking (at 3 cm/s) prior to an experimental noxious stimulus or clinical heel lance significantly reduced noxious-evoked brain activity in term neonates compared to non-CT-optimal stroking. We replicated

this study in an independent sample of term neonates and showed consistent results and a similar effect size in the group receiving the stroking intervention (Cobo et al., 2021). However, in this study stroking was delivered by the researcher using a soft experimental brush with a known force. Although the study did not identify a significant effect of the intervention on a behavioural pain score, it was not powered to investigate this. Considering CT-optimal stroking is a natural parental behaviour and there is evidence to suggest CT fibres respond optimally to touch at human skin temperature (Ackerley et al., 2014), hands-on parental stroking has the potential to provide even greater benefit than CT-optimal brushstrokes. It is hoped that this clinical trial would validate the results of this small non-randomised study and crucially translate the experimental paradigm into a simple and effective hands-on parent-led intervention.

2.2 Objectives

Table 1 Objectives and outcome measures. EEG: Electroencephalography, PIPP-R: Premature Infant Pain Profile – Revised, STAI-T: State-Trait Anxiety Inventory-Trait, STAI-S: State-Trait Anxiety Inventory-State.

Objectives	Outcome measures
Primary objective	**Primary outcome measure**
(i) To test whether parental touch prior to the clinically required heel lance reduces noxious-evoked brain activity assessed using EEG following a heel lance.	(i) Magnitude of noxious-evoked brain activity following a heel lance (EEG data recorded in the 1000 ms period following each heel lance).
Secondary objectives	**Secondary outcome measures**
(i) To test whether parental touch prior to the heel lance reduces clinical pain scores (PIPP-R) following a heel lance.	(i) PIPP-R score during the 30 s period post-heel lance.
(ii) To test whether parental touch prior to the heel lance reduces incidence of tachycardia activity following a heel lance.	(ii) Tachycardia in the 30 s period post-heel lance.
(iii) To test whether parental touch prior to the heel lance reduces parental anxiety, compared with post-procedural touch.	(iii) STAI-S scores post procedure.

3 Section 3: Study methods

3.1 Trial design

This is a multicentre, individually randomised, parallel-group interventional trial, with two research sites (John Radcliffe Hospital, Oxford, UK, and Royal Devon and Exeter Hospital, Exeter, UK). In this study, we aim to determine whether parental touch prior to a painful clinical procedure (heel lance) provides effective analgesia.

Participants will be studied on a single test occasion while they are in hospital, when they require a heel lance as part of routine clinical care. **No extra blood tests or noxious procedures will be performed for the purpose of the study.** A sham heel lance will be administered to all participants on the same heel as the heel lance, prior to the actual heel lance. The sham heel lance procedure is not a blood test and not noxious. The lancet is placed against the baby's foot but angled away – upon release, the blade is fired into the air rather than into the foot. This is to simulate the experience of having a blood test without the noxious input – i.e. the baby's foot being held by the clinical researcher, the feel of the lancet placed against the foot, and the click sound and tactile sensation when the button is pressed and blade released. Brain activity is recorded in response to the sham heel lance in addition to the clinical heel lance to ensure that we are assessing responses to the noxious aspect of having a blood test. This is an important validity check for the integrity of our recording measures.

Recordings used for outcome measures include an EEG, pulse oximetry, ECG, and facial video recording. Participants will be included in the study for approximately an hour. This will be approximately 30 min before and after the time when the heel lance is performed. Each participant will only be included in the trial once. If a participant requires additional heel lances as part of routine clinical care, to acquire sufficient blood for clinical assessment at the time of the test occasion, we will also record the responses to this. The research monitoring equipment will not be removed from the participant between heel lances (if more than one heel lance is required for clinical purposes) in order to ensure we do not interfere with the clinical procedures. For analysis, we will only consider the first skin-breaking heel lance.

3.2 Intervention

Parental touch in the form of a stroking intervention is performed at approximately 3 cm/s for 10 seconds down the lower limb receiving the heel lance. The parents are trained verbally by a researcher on how to perform the intervention. During the intervention, the parent will follow an animation which shows the correct speed of stroking, and which is repeated three times, reflecting the number of times the participant's leg will be stroked. The same animation is shown during training as well. The same animation will be used to train parents and to guide their stroking in both trial arms.

3.3 Randomisation

This trial has an intervention and control group. Randomisation of participants to each group will be managed via a secure web-based randomisation facility provided by Sealed Envelope Ltd, UK. Participants will have a roughly equal chance of being allocated to either arm - the randomisation program will use a minimisation algorithm to ensure approximate balance

between the groups with respect to five minimisation variables: postmenstrual age at birth, postnatal age at time of randomisation, sex, the primary indication for blood sampling, and centre. A minimisation algorithm was selected due to the relatively small sample size and the need to balance multiple baseline covariates known to be important. The minimisation algorithm incorporates a random element (Sealed Envelope Ltd., 2022). The allocation sequence will be concealed: the users of the system will have no insight into the next allocation. Participants will be randomised as soon as parental consent has been provided.

3.4 Sample size and power

The assumptions for these calculations are based on data from previous studies investigating the effect of (experimenter-led) soft brushing of the skin at CT-optimal rate on the response to a noxious stimulus or clinical heel lance in term neonates (Cobo et al., 2021; Gursul et al., 2018). The mean brain activity evoked by heel lancing in the control group is estimated to be 1.07 with a standard deviation (SD) of 0.66. We consider a 40% reduction in the intervention group to be clinically significant based on similar reductions in verbally reported pain scores in adults (Dworkin et al., 2008), and similar reductions of noxious-evoked brain activity in adults corresponding to significantly reduced pain scores (Lorenz et al., 1997; von Mohr et al., 2018). A 40% reduction is in line with our previous studies investigating CT-optimal brushing prior to a heel lance, where we observed a 40% (Gursul et al., 2018) and a 39% (Cobo et al., 2021) reduction in the brushed group compared with the control group.

For sample size planning, the intervention arm evoked magnitude mean is 1.07 and SD is 0.66; the control arm evoked mean magnitude is 0.642 and SD is 0.66; which results in a Cohen's D effect size of 0.648. With 90% power, a two-sided 5% significance level, and an allocation ratio of 1, we estimated a sample size of n=102 infants. Allowing for approx. 10% loss due to technical difficulties or clinical issues, the final sample size was estimated to be n=112.

3.5 Framework

This is a superiority trial comparing standard of care and parental stroking performed before a heel lance to standard of care and parental stroking performed after a heel lance.

3.6 Statistical interim analysis

No interim statistical analysis is planned.

3.7 Timing of final analysis

After data from the final participant have been collected, the SAP will be published online. Only after SAP publication, will the data be unblinded and analysis performed.

3.8 Timing of outcome assessments

The timing of outcome assessment will vary depending on the outcome. An overview is presented under 'Recordings' in Figure 1.

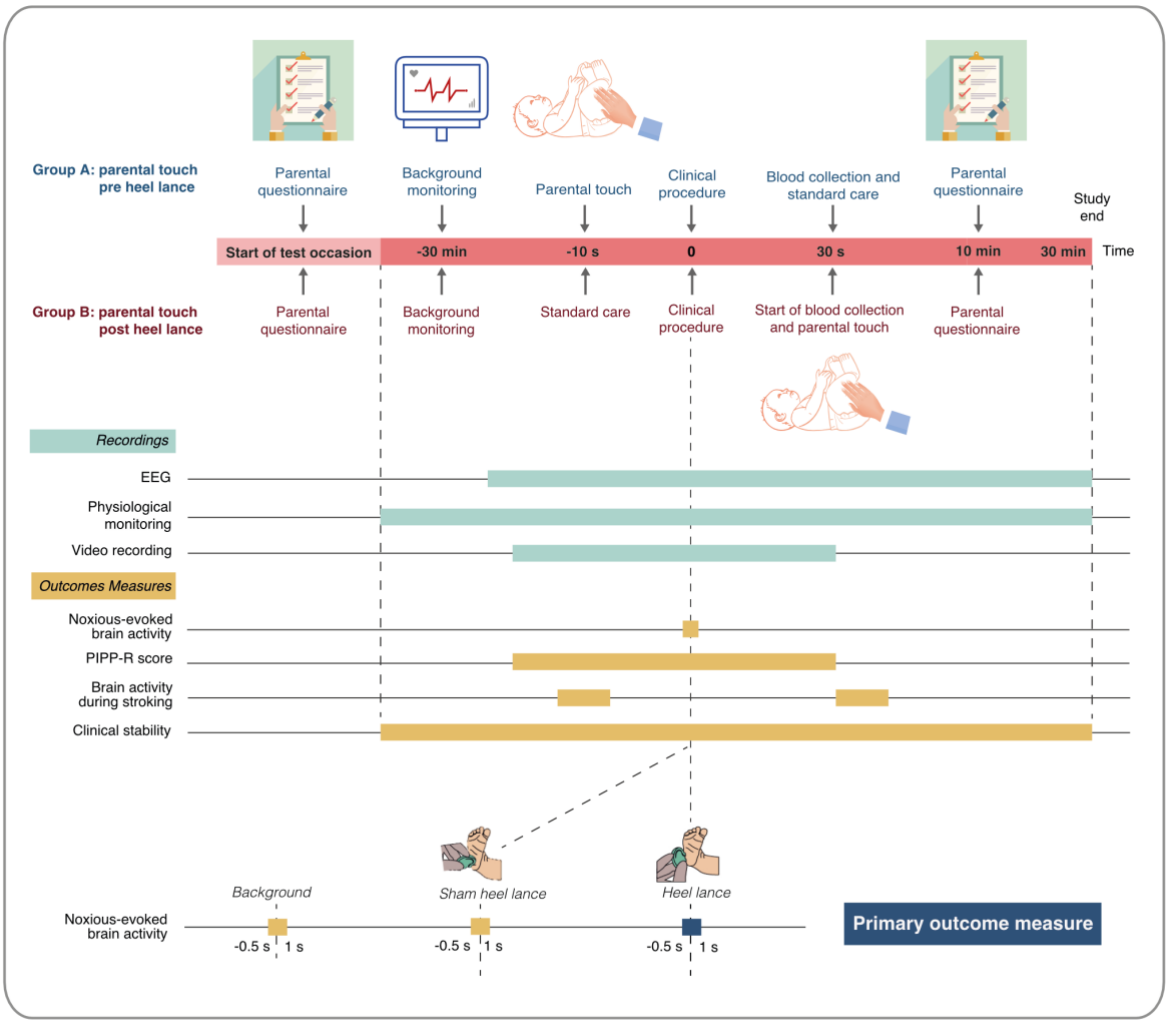

Figure 1 Timings of outcome recordings and outcome measures. EEG: electroencephalography, PIPP-R: Premature Infant Pain Profile-Revised.

At least 30 minutes before the sham heel lance and heel lance (and after randomisation), the research team will set up the electroencephalography (EEG), physiological recording (electrocardiogram (ECG) – for heart rate and respiratory rate; pulse oximeter – for oxygen saturations), and video recording (for facial expression change). Physiological monitoring will continue from approximately 30 minutes prior to the sham heel lance and heel lance until approximately 30 minutes afterwards. Noxious-evoked brain activity (measured using EEG) will be recorded for a minimum of 10 minutes prior and 10 minutes after the sham heel lance and heel lance. Video recording will continue from approximately 30 seconds before to 30 seconds after the sham heel lance and heel lance. The neonate's foot will be held by the clinical researcher during the video recording for PIPP-R scoring, the sham heel lance stimulus and the heel lance. The foot will be held in the same manner in both trial arms.

Clinical stability of the neonates will be assessed throughout the trial period using the physiological recordings. These measures will be calculated from oxygen saturation recordings, and from ECG recordings to monitor heart rate and the impedance pneumograph to monitor respiratory rate. These data will be monitored and recorded on our data logging

equipment for approximately 30 minutes before and 30 minutes after the sham heel lance and heel lance.

Parental questionnaires will be recorded as outlined in Table 2.

Table 2 Trial parental questionnaires, timing of administration.

Questionnaire section	**Topic**	**Timing of administration**	**Questionnaire administrator**
20-point State-Trait Anxiety Inventory (STAI)-T	Trait anxiety	Start of test occasion	Administered verbally by researcher
20-point State-Trait Anxiety Inventory (STAI)-S	State anxiety pre-heel lance State anxiety post-heel lance	Start of test occasion After the procedure and intervention are completed	Administered verbally by researcher Administered verbally by researcher
Four-point distress questionnaire	Emotional constructs experienced at time of the clinical heel lance	After the procedure and intervention are completed	Administered verbally by researcher
Anonymous survey	Views on the trial and infant research	End of test occasion	Completed by parent

4 Section 4: Statistical Principles

4.1 Confidence intervals and P values

The level of statistical significance will be set at 5% for the primary outcome. The level of statistical significance for all secondary outcomes will be set at 5% overall. For secondary outcomes, multiple comparisons will be corrected with the Holm method. We will report effect sizes and 95% CI for all outcomes.

4.2 Adherence and protocol deviations

Adherence to the intervention will be assessed qualitatively during the trial by observing the parent while stroking the infant. Parents will be trained on how to apply the intervention and will receive feedback from the research team during training. During training and while delivering the intervention, they will be guided by an animation displaying an empty bar which is progressively filled at 3 cm/s. This bar is filled three times in a row, for a total of 10 s. The animation therefore clearly shows start and end times of the intervention, as well as the speed at which the stroking should be performed and that the stroking is performed three times in a row. The use of an animation to train and guide the parents helps to ensure training and guidance is standardised across participants. In the intervention group the protocol will be considered as adhered to if the start of the parental stroking commenced less than 45 seconds before the heel lance. Deviations from the assigned intervention will be reported alongside the results. If a participant is withdrawn from the study, the reason for withdrawal will be reported. Furthermore, we will report the timing of withdrawal in relation to communicating to parents their child's allocation arm and in relation to the trial's timeline wherever possible.

4.3 Analysis populations

Participants are randomised to being stroked either before or after the heel lance. Standard of care will be provided to all infants enrolled in the trial. At the time of the heel lance, which is when the primary outcome measure is recorded, participants randomised to the intervention arm will additionally have been stroked prior to the heel lance, while participants randomised to the other arm will not have been stroked yet. To assess the effect of the intervention the per protocol analysis set will be used. Questionnaire data will be included in the per protocol analysis irrespective of the timing of the stroking intervention.

5 Section 5: Trial Population

5.1 Screening and recruitment

The number of participants whose parents were approached will be recorded and compared to the number of participants included in the trial.

5.2 Eligibility

Inclusion criteria:

- Participants born at the John Radcliffe Hospital, Oxford or the Royal Devon and Exeter Hospital, Devon
- Neonates born at or after 35+0 weeks gestation
- Neonates with a postnatal age of ≤7 days
- Neonates who require a heel lance as part of clinical care
- Neonates for whom parents/guardians have given written informed consent for participation.

Exclusion criteria:

- Hypoxic Ischaemic Encephalopathy
- Intraventricular haemorrhage (IVH) > grade II
- Received any analgesics or sedatives in the last 24 hours
- Congenital malformation or genetic condition known to affect neurological development
- Born to mothers who have a history of substance abuse.

5.3 Withdrawal/Follow-up

Parents may withdraw their neonate from the trial at any time and they are not obliged to give a reason. If parents choose to withdraw their child after the study has begun, they will be asked whether data already collected may be retained and used for the purposes of the trial. Parents will be made aware that this decision has no impact on any aspects of their infant's continuing care. The attending clinician may withdraw the neonate from treatment if they consider this to be in the best interest of the neonate's health and well-being. If any of the exclusion criteria manifest after consent but prior to data collection, the participant will be withdrawn.

Withdrawals will be included in the CONSORT diagram. The timing and reason (where available) for withdrawal will be recorded.

5.4 Baseline patient characteristics

We will report baseline patient characteristics. The list of baseline characteristics will include:

- Gestational age at birth (weeks)
- Postmenstrual age at time of study (weeks)
- Postnatal age at time of study (days)
- Birthweight (g)

- Sex
- Mode of delivery
- Apgar score at 1 min
- Apgar score at 5 min
- Primary reason for blood test
- Estimated cumulative prior pain exposure

Baseline characteristics will be presented by randomisation arm.

6 Section 6: Analysis

6.1 Outcome definitions

6.1.1 Primary outcome

The primary outcome measure is the magnitude of noxious-evoked brain activity following a heel lance (EEG data recorded in the 1000 ms period following each heel lance). EEG traces at the Cz electrode will be bandpass filtered from 0.5—33.75 Hz. The data will be Woody filtered with a maximum jitter of ± 100 ms in the region of 400—700 ms after the stimulus by identifying the maximum cross-correlation with the previously defined template of noxious-evoked brain activity (Hartley et al., 2017). The template of noxious-evoked brain activity will then be projected onto each individual trial in the region 400—700 ms after the stimulus to calculate the magnitude of the noxious-evoked brain activity. Individual EEG traces will be rejected if the point of stimulation is not marked on the EEG recording due to technical failure or if there is artefact (for example, gross movement artefact, electrical interference) during the immediate baseline or post-stimulus period. Participants and personnel involved in primary outcome data collection cannot be blinded to trial arm. However, the primary outcome measure is a pharmacodynamic biomarker thus recording the outcome is not influenced by participant or researcher judgement. The primary outcome measure will be extracted using automated scripts. Artefact identification and rejection involves subjective assessment. This task will be performed by two blinded investigators, with any discrepancies in assessment resolved by discussion.

6.1.2 Secondary outcomes

6.1.2.1 PIPP-R

Clinical pain scores will be calculated using the Premature Infant Pain Profile - Revised (PIPP-R)(Stevens et al., 2014). The score is composed of postmenstrual age, behavioural state, physiological changes (heart rate and oxygen saturation changes), as well as facial expression duration (brow bulge, eye squeeze and nasolabial furrow)(Stevens et al., 2014). The range of PIPP-R score is 0—21. The postmenstrual age at the time of the study will be calculated automatically based on the gestational age at birth and postnatal age on the secure trial electronical database where the test occasion details are stored. For each participant, video recordings of the infant's facial expressions during sham heel lance and heel lance will be acquired. These videos will then be clipped for scoring purposes so that, in the end, they will be composed of 15 seconds baseline before the intervention (stroking pre heel lance arm) or before the heel lance (stroking post heel lance arm), the moment of the heel lance, and the 30 seconds response thereafter. To prevent the scorer from differentiating between trial arms or between sham heel lance and heel lance, a short fragment of the video lasting up to a few seconds will be cut out between the baseline and procedure in both trial arms and for both sham heel lance and heel lance. Therefore, the clipped videos will briefly skip forward just before the sham heel lance or heel lance in both trial arms. The clipped videos will last 45 s each and will be assigned random numbers. For each participant, researchers who have not been involved in the specific test occasion will score the facial expressions' duration and the behavioural state remaining blind to trial arm and stimulus type, i.e. sham heel lance or heel lance. The facial expression and behavioural state scores will then be entered onto the database. The vital signs data will be downloaded directly from the vital signs monitor and

physiological changes will be automatically quantified via a bespoke MATLAB script. Physiological traces will be assessed for quality by a researcher not involved in the specific test occasion and blinded to arm allocation. The physiological values will be entered onto the database as well. The calculation of the total PIPP-R score for each participant for sham heel lance and heel lance is performed automatically on the database. All PIPP-R scores will be calculated prior to unblinding the data for statistical analysis.

6.1.2.2 Post-procedural tachycardia

The tachycardia outcome per infant will be dichotomous (i.e. 'yes/no' per infant). Tachycardia will be defined as an increase in heart rate over 160 bpm in the 30 s period post heel lance if the mean heart rate in the baseline period is less than 160 bpm. The baseline period is defined as the 15 s period prior to the start of the stroking. In the control group this was a sham marker. This will be automatically assessed with a bespoke MATLAB script. All tachycardia outcomes will be assessed prior to unblinding the data for statistical analysis.

6.1.2.3 Parental questionnaires (STAI)

The State-Trait Anxiety Inventory (STAI) is a widely used, validated and reliable assessment for anxiety (Spiegelberger et al., 1983). The STAI has a trait (STAI-T) and a state version (STAI-S). The STAI-T scale consists of 20 statements exploring general feelings of anxiety. The STAI-S scale also consists of 20 statements and requires subjects to rate their anxiety at a particular moment in time. The STAI-S scale can be used to determine the level of anxiety induced by stressful procedures, whereas the STAI-T is used to identify general levels of anxiety. Each question is rated on a 4-point Likert scale. The scores range from a minimum of 20 to a maximum of 80 for both the STAI-T and STAI-S subscales, with higher scores indicating higher anxiety levels.

STAI-S scores post-procedure will be compared between trial arms. The parent delivering the intervention will be blinded to trial arm allocation at the time of answering the STAI-T and STAI-S questionnaires before the heel lance. A researcher not blinded to trial arm will verbally ask the questions and will read the possible multiple-choice answers (not at all/somewhat/moderately/very much) to the parent. The parent will be informed about their infant's allocation to a trial arm after they have answered both STAI-T and STAI-S prior to the heel lance, so that answers to these initial questions will not be influenced by knowledge of their infant's trial arm allocation. The second set of questions (STAI-S, four-point distress questionnaire) is asked after the parent has delivered the stroking intervention to their infant; therefore, the parent will be unblinded to trial arm at this point so that the questionnaires can assess how the intervention influences parental anxiety. The answers will be entered on an electronic database where the questionnaires scores will be calculated automatically.

6.1.3 Exploratory outcomes

The exploratory outcome measures are stated in the protocol. Statistical analysis of these outcomes will be detailed in separate reports.

6.2 Analysis methods

6.2.1 Primary outcome

The magnitude of noxious-evoked brain activity will be compared between two groups using multiple linear regression analysis. In the regression model, the group allocation variable will be adjusted for the five minimisation variables.

We will assess the relevant assumptions for our chosen statistical test. Given our design, model, and variables of interest, we do not expect issues with linearity, independence of residuals, or exogeneity of variables. For large deviations from normality, we will consider linear regression via permutation testing. For large deviations from homoscedasticity, we will consider a robust standard error linear regression. We will present the mean, SD, and 95% CI for the effect size of interest, i.e., the difference in mean magnitudes of noxious-evoked brain activity between groups.

6.2.2 Secondary outcomes

6.2.2.1 PIPP-R

PIPP-R scores will be compared between the two groups using multiple linear regression analysis. In the regression model, the group allocation variable will be adjusted for the five minimisation variables.

We will assess the relevant assumptions for our chosen statistical test. Given our design, model, and variables of interest, we do not expect issues with linearity, independence of residuals, or exogeneity of variables. For large deviations from normality, we will consider linear regression via permutation testing. For large deviations from homoscedasticity, we will consider a robust standard error linear regression. We will present the mean, SD, and 95% CI for the effect size of interest, i.e., the difference in mean PIPP-R scores between groups.

6.2.2.2 Post-procedural tachycardia

Tachycardia will be compared between two groups using multiple logistic regression analysis. In the regression model, the group allocation variable will be adjusted for the five minimisation variables and a baseline estimate of pre-procedural heart rate.

We will assess the relevant assumptions for our chosen statistical test. Given our design, model, and variables of interest, we do not expect issues with linearity between log odds ratio and explanatory variables, independence of residuals, or exogeneity of variables We will further ensure that we have no perfect separation. We will present the odds ratio with a 95% CI.

6.2.2.3 Parental questionnaires (STAI-S)

The STAI-S scores will be compared between two groups using multiple linear regression analysis. In the regression model, the group allocation variable will be adjusted for the five minimisation variables and a baseline estimate of pre-procedural anxiety (pre-procedural STAI-S).

We will assess the relevant assumptions for our chosen statistical test. Given our design, model, and variables of interest, we do not expect issues with linearity, independence of residuals, or exogeneity of variables. For large deviations from normality, we will consider linear regression via permutation testing. For large deviations from homoscedasticity, we will consider a robust standard error linear regression. We will present the mean, SD, and 95% CI for the effect size of interest, i.e., the difference in mean STAI-S scores between groups.

6.3 Missing data

Missing data will occur in our trial due to, for example, equipment failure, artefacts within the EEG recording, or clinical care changes if, for example, the heel lance is not deemed necessary anymore by the clinical team after a participant has already been enrolled. We will assess the impact of missing data on our results using the approaches outlined in section 6.2.3.

6.3.1 Sensitivity analyses

For both primary and secondary outcomes, we will assess the role of missingness. We will describe:

1. The reasons for missing outcomes, wherever possible
2. The extent of missingness by trial arm
3. The extent of missingness by covariates that might influence the missing variable or the pattern of missingness.

For the primary outcome only, we will do the following sensitivity analyses:

1. Multiple imputation to assess sensitivity to data missing at random
2. Pattern mixture model to assess sensitivity to data missing not at random

6.4 Additional analyses

We will assess and report intra- and inter-rater reliability for the PIPP-R ratings. We will use the intraclass correlation coefficient and a one-way model to assess rater consistency. Reliability will be assessed on the total PIPP-R score only and for heel lances and sham heel lances separately.

6.5 Harms

A summary of safety data including severe adverse events causally linked to trial participation will be included in the results.

6.6 Statistical software

R or MATLAB will be used for statistical analysis. Information on the packages used will be reported.

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8 Approval

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9 Document history

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