The effect of Regional Anaesthesia and Genetic Factors on the development of Chronic Pain following Knee Arthroplasty

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1.1 Introduction

This research will be a randomized, controlled, non-blinded study, with analysis of both clinical and genetic outcomes. The tools used during the study, such as questionnaires as the WOMAC® and SLANSS, have been validated before. The analysis will be quantitative, using validated methods already used in other research projects.

1.2 Sample size

A literature review showed that the WOMAC® score before surgery ranges from 35 to 49, for a total of 96. Following surgery, this range decreases to 12.6 to 18.4, with a change of 21.5 to 30.6 from the pre-operative WOMAC® score. (Kahn, Soheili and Schwarzkopf, 2013; Wylde *et al.*, 2015)

It is calculated that for a difference of 10 units between the WOMAC® scores in the two groups, eighty patients per trial arm would be required for a power of 80%. To allow for patients lost to follow up and exclusions, a total of 200 patients are to be included in this research project.

1.3 Ethical Approval

Approval from the various orthopaedic consultant surgeons caring for the patients involved in the trial was obtained. After clearance from the Data Protection Unit at Mater Dei Hospital was sought, the University Research and Ethics Committee was asked to approve the study.

Ethics approval was obtained in May 2017, with the reference number 05/2017.

1.4 Recruitment

All patients who are to receive a Total Knee Replacement (TKR) under the care of the participating orthopaedic firms, will be identified from the elective surgical lists. Initial screening for suitability of the patients was done by inspecting the preoperative assessment sheet. This is usually done at the Preoperative Assessment Clinic at Mater Dei Hospital some weeks before the surgery.

Patients will be excluded if:

- Age is more than 75 years
- Second TKR during the study period

- Rheumatoid arthritis as cause for TKR
- Revision TKR
- Any contraindication to any of the study drugs, including paracetamol, codeine or diclofenac
- Any contraindication for spinal anaesthesia
- Pre-existing evidence of chronic pain syndromes, such as fibromyalgia

Suitability will then be confirmed by interviewing the patients before enrolment is considered.

Recruitment starts in April 2017, and is expected to last for 2 – 3 years.

1.5 Consent

Consent will be obtained from each patient after the nature of the research is explained to the patient. Possible participants will be also informed of any requirements that would be additional to normal clinical practice, such as blood letting, post-operative visits, and telephone questionnaires.

Patients who agreed to participate will be given the opportunity to ask any questions and written informed consent will be obtained. Patients will be also informed that they have the option to withdraw from the trial at any stage. In such cases, data from such patients will not be used in the analysis. The signed consent forms will be kept until the research project has ended.

1.6 Randomization

Randomization will be performed using a minimization method, as described by Pocock and Simon. (Pocock and Simon, 1975) Normal randomization may lead to imbalances between the control and the intervention group, especially in smaller groups. Minimization calculates the imbalances before the patient is allocated to either group, and the allocation which leads to the least imbalance is then chosen. This was done in order to optimize matching between the two study groups, given the relatively small size of subjects in each arm.

A web-based application, coded by the principal investigator in PHP 5.6 and MySQL 8.0, will be used for such randomization. This was done using a secure platform, with access limited only to the investigators. The randomization will allocate patients to either a spinal

anaesthetic (Group SP) or a general anaesthetic with a femoral block (Group GA) depending on five stratifications:

- Age
- Gender
- BMI
- Surgical Firm
- Pre-operative WOMAC

For each stratification factor, the number of patients previously assigned in either group will be calculated. The difference between these totals is the imbalance score, and the imbalance score of each factor will be added together to give the total imbalance score.

The patient will be then assigned to the group with the smallest marginal total, in order to reduce the total imbalance score. This method has been previously described by Taves. (Taves, 1974; Scott *et al.*, 2002)

In cases where the difference will be the same, the randomization was then done by chance, with a 70% chance of being allocated to the spinal group. This will be done in anticipation of a greater amount of crossover from the spinal group to the general anaesthesia group.

1.7 Treatment Arms

In all cases, subjects will follow a standard analgesic protocol that had been established by the researcher in prior studies.

All subjects will receive Paracetamol 1g and Diclofenac 50mg orally, preoperatively upon being prepared for transfer to theatre. Furthermore, all enrolled patients will be prescribed post-operative analgesia. This which consisted of:

Morphine as Patient Controlled Analgesia (PCA)	1mg boluses and a lockout time of 5 minutes
Paracetamol	1g orally every six hours
Codeine	30mg orally every eight hours
Diclofenac	50mg orally every eight hours
Ondansetron	4mg – 8mg intravenously when needed

The following day, the Morphine PCA will be changed to oral Morphine (OroMorph®). The first 10mg dose will be given in the morning, and then again every four hours if required.

These will be prescribed on the prescription chart by the investigators, in order to minimise errors.

1.7.1 GA group

Subjects randomized to the General Anaesthesia group (GA) will receive a standard general anaesthetic regime as per current practices. This would include:

Induction	Propofol titrated to effect Fentanyl, at 1 – 2 µg / kg If necessary, Muscle relaxant of choice
Maintenance	Sevoflurane, adjusted to maintain depth of anaesthesia
Analgesia	Morphine or Pethidine as required Femoral nerve block
Femoral Nerve Block	Ultrasound-Guided Femoral nerve, with Bupivicaine dose set by anaesthetist

Any long-acting opiate analgesics that will be administered during the procedure were included in the total morphine consumption. Shorter acting drugs, principally Fentanyl, will not included in this total, since these would not have an impact on pain relief for more than thirty minutes.

1.7.2 SP group

Subjects that will be randomized to the Spinal Anaesthetic group (SP) will receive an intrathecal injection of 0,5% Heavy Bupivacaine, at a dose set by the anaesthetist, and Diamorphine, at a recommended dose of 300µg.

The intrathecal injection was performed by the attending anaesthetist, as per current recommendations.

1.7.3 Cross-over between groups

In cases where the caring anaesthetist feels that the randomization will not be appropriate on clinical grounds, or whenever a spinal anaesthetic cannot not be administered, then cross-over to the other group will be allowed.

Given that it had been decided to perform statistical analysis on a per-protocol basis, patients who cross over to the other group will be then included in that group, and not excluded from the trial.

1.8 Blinding

Due to the nature of the treatment, it will be not possible to blind either the patient or the clinician to the treatment. Neither will it be possible to blind the investigators collecting the data in the peri-operative phase of the research project, since it will be necessary to identify any patients who would cross over to the other group.

However, care will be taken to limit the exposure of the treatment provided when collecting data at later stages of the study. For instance, during the telephone interviews, the investigators will only have access to patient name, the date of surgical procedure and the telephone number. This will be done in order to avoid bias during the conduct of the telephone questionnaires.

1.9 Data Collection

Investigators will collect data at baseline, the day following surgery, and at three months and six months after surgery.

This will be collected on a web platform on a secure server used specifically for the project. This was programmed by the principal investigator, using a MySQL database and a PHP backend, and optimized for use on a mobile phone. The application was designed to allow limited exposure of data collected to the investigators, both for Data Protection and for blinding purposes.

Upon registration of the subject into the system, a unique four-digit reference number will be generated and assigned to each entry. This will be used to anonymise blood samples that will be taken, and also to label the genetic samples.

1.9.1 Baseline

Once enrolled, demographic data will be collected. This includes:

- Name, Surname, ID Numbers (for identification purposes only)
- Age
- Body Mass Index
- ASA
- Telephone number(s) of the patient
- Date of surgical procedure
- Surgeon responsible for the procedure
- Baseline questionnaires will be used to assess pain, function, disability and incidence of neuropathic pain. These will be the:
    - Western Ontario and McMaster Universities Osteoarthitis Index (WOMAC®) - score for pain, function and disability[1]
    - Self-reported Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS) – score for assessment of neuropathic pain

The WOMAC® and S-LANSS questionnaires will be obtained in English, and then translated into Maltese. The actual version used depends on patient preference, but the English version will be used as reference when any clarification was required.

1.9.2 Early Postoperative Period

On the morning following the surgery, an investigator will visit the patient. The anaesthetic that will be been administered during the procedure will be confirmed. An analysis-by-protocol had been agreed a priori, so changes in the anaesthesia from the randomization will be marked as crossover cases.

The investigator will then collect data on:

- The current intensity of pain at rest, before mobilisation, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The intensity of pain during physiotherapy, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)

[1] Licensed from Prof N Bellamy, Australia

- Morphine consumption, including intra-operative and PCA consumption, for the first 24 hours
- The intensity of any nausea, on a scale of 0 to 4 (0: no nausea, 1: mild, 3: moderate, 4: severe)
- Any vomiting episodes, and if any antiemetics were given

If other long-acting opiates besides morphine will be used, namely pethidine, then these were converted to morphine equivalents. Furthermore, oral morphine will be converted to intravenous equivalents assuming a 50% bioavailability.

1.9.3 Assessment at 3-months

Three months after surgery, all subjects will be interviewed using a phone call. The investigators will collect the following data:

- The intensity of pain at rest, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The intensity of pain on exercise, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The use of any analgesic during the previous two weeks
- S-LANSS
- WOMAC®

1.9.4 Assessment at 6-months

Six months after surgery, all subjects will be interviewed using a phone call. The investigators will collect the following data:

- The intensity of pain at rest, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The intensity of pain on exercise, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The use of any analgesic during the previous two weeks
- S-LANSS
- WOMAC®

1.10 Genetic Sampling

1.10.1 Blood sampling

After enrolment, a sample of blood will be taken during anaesthesia, to avoid patient discomfort. A total of 3mL will be collected in an EDTA sample bottle, and labelled with a unique four-digit number to ensure blinding. This sample will be stored in a temperature-monitored refrigerator at 4ºC to 8ºC, then transported to the Biomedical Sciences Building, at the University of Malta, to be stored in the cold rooms at 4ºC.

1.10.2 Preparation, DNA extraction

The blood samples will be allowed to warm to room temperature, then will be properly mixed before DNA extraction commenced. Each will be then given a sequence number, so as to further blind the investigator.

A rapid DNA extraction kit was chosen. This was the Qiagen® DNeasy Blood & Tissue Kits (Qiagen GmbH, Dusseldorf, Germany). This kit utilises proprietary reagents to lyse cells, purify and extract DNA from a sample of blood. A silica-based filter is used to adsorb the DNA until the final stages of the process. The yield from such a process is slightly less than a salting-out method, but at a fraction of time. (Maurya, Kumar and Sundar, 2013) It also needs a much smaller blood sample, in the order of 100µL compared to 1mL for the salting out method.

The process used follows the recommended procedures by the company, with some minor modifications:

1. 80 µL of Phosphate-buffered Saline, 20 µL of Proteinase K will be mixed with 120 µL of blood in a 1.5mL microcentrifuge tube, for a total volume of 220 µL. To this, 200 µL of Buffer AL solution will be added, and the solution will be then mixed by vortexing for 5 – 10 seconds.
2. This mixture will be then incubated at 56ºC for 11 minutes. 200 µL of pure ethanol will be added to the mixture, which will be then mixed thoroughly by gentle shaking.
3. This mixture will be transferred to the DNeasy mini spin column, and centrifuged at 6000 x g for 1 minute 15 seconds. The resulting flow-through will be discarded, and the spin colum placed in another 2 mL microcentrifuge tube.

4. 500 µL of Buffer AW1 solution will be placed in the spin column, and centrifuged at 6000 x g for 1 minute 15 seconds. The flow-through will be discarded.
5. The process will be repeated with 500 µL of Buffer AW2 solution, and centrifuged at 20,000 x g for 3 minutes 15 seconds.
6. To remove as much ethanol as possible, the spin column will be then centrifuged with no solution at 20,000 x g for another 1 minute 15 seconds.
7. A volume of 30 - 50 µl of Buffer AE will be used to elute the DNA. This volume will be pipetted into the spin column, and left to stand for 5 minutes.
8. The spin column will be centrifuged at 6000 x g for 1 minute 15 seconds, and the eluate will then be collected in a 1.5 mL microcentrifuge tube suitably labelled.

The sample will be then checked for DNA concentration and purity using a Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, US). This uses UV light to measure absorbance of a sample, and depending on the ratio of absorbance at 230 nm, 260 nm and 280 nm wavelengths, it measures the concentration of DNA.

Aliquots containing 30 µL of sample DNA solution at a concentration of 25 µg will be prepared for each sample. This will be done to standardize each sample for use with the realtime PCR. These were stored at –20^{o}C until further use.

Blood that will not be used was stored at -20^{o}C in the cold storage rooms in the Biomedical Sciences Building, University of Malta.

1.10.3 DNA analysis

Genetic analysis, to determine the presence of the investigated SNPs, will be performed using quantitative polymerase chain reaction (qPCR), also known as realtime PCR. During a qPCR, a sequence of a sample DNA is amplified (amplicon) using specific primers, and this reaction is performed repeatedly.

For this research, TaqMan™ probes will be used. By quantifying the threshold at which the fluorescent intensity is above a control, the cycle threshold, Ct, is calculated. This is then used to show the presence or absence of a SNP.

The procedure used is described below:

1. The aliquots containing DNA will be thawed.
2. A master mix with TaqMan™ Universal Master Mix II, with UNG (Thermo Fisher Scientific, US), and the TaqMan™ predesigned SNP Genotyping Assay will be prepared.
3. The required amount was pipetted to 0.1 mL strip tubes, which will then be individually labelled.
4. One μL of DNA sample will be added to the mixtures, with one strip tube designated as the Negative Template Control.
5. qPCR will be then performed using the Rotor-Gene Q (Qiagen GmbH, Dusseldorf, Germany). The sequence used was recommended by the probe assay manufacturer
6. The Rotor-gene Analysis Software, version 2.4.1 (Qiagen GmbH, Dusseldorf, Germany) was used for the analysis the results of each realtime PCR run. The threshold was set manually for each channel, depending on the Negative Template Control. (**Error! Reference source not found.**)
7. A Scatter plot analysis was then used to identify the genotype of each sample
8. The data was then exported to a comma separated spreadsheet for further analysis.

1.11 Statistical analysis

The primary outcomes for the study will be the Numerical Rating Score during physiotherapy in the early phase, and the change in WOMAC® Pain subscore at three and six months from the preoperative value.

Secondary outcomes will be also considered. During the acute phase, the secondary outcomes investigated will be acute pain, as measured by the Numerical Pain Scales at rest, and morphine consumption. For the latter stages of the study, the WOMAC® score, the pain part of the WOMAC® score, the S-LANSS at three and six months, and the improvement in WOMAC® score at three months and at six months will be considered. The incidence of chronic post-surgical pain (CPSP) at three and at six months, defined as the number of patients with a low WOMAC® pain subscore (less than 5 out of a total of 20), will also be considered.

A p-value of 0.05 will be taken as significant. Given the strong possibility of having a considerable amount of cross-over, an analysis by protocol, rather than by intention to treat, will be considered. In order to reduce bias, a further analysis by intention to treat will be performed, to validate the primary results.

Statistical analysis will be performed with R (version 3.5.1), using R Studio (Version 1.1.442). Univariate analysis will be performed initially, using parametric or non-parametric tests where appropriate. The data will be first checked for normality and skewness using visual methods, and other tests such as Shapiro's test of normality. When appropriate, t-tests, Mann-Whitney U tests, Kruskal-Wallis test and chi-squared tests will be used for univariate analysis. Linear regression, polynomial linear regression and logistic regression (as part of generalized linear models) will be used for multivariate analysis when indicated. For such analysis, any factors found to have a p value of 0.25 or less during univariate analysis will be used as predictive variables in such models, unless otherwise stated. (Zhang, 2016) A stepdown modelling will be performed, with the predictive variables with least significance being dropped during each iteration. (Calcagno and de Mazancourt, 2010)

Statistical analysis using the WOMAC® score will be performed using non-parametric tests. It is not necessary to treat the WOMAC® score as a categorical variable, but rather, as an ordinal variable. Hence, it will be possible to compare median scores between two samples.