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Knee replacement surgery significantly elevates the urinary inflammatory biomarkers neopterin and 7,8-dihydroneopterin

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ABSTRACT

Context: Knee arthroplasty surgery is significant trauma, leading to an activated immune system causing inflammation and oxidative stress. Many current biomarkers are invasive, costly, and often slow to analyse, limiting their use for rapid inflammatory measurements.

Objectives: We have examined the use of urinary neopterin and total neopterin in knee arthroplasty patients to non-invasively measure oxidative stress and inflammation from immune system activation. We aim to validate the use of these biomarkers for quick, cost effective and predictive measurements of post-surgical inflammation assessment.

Methodology: 19 Knee arthroplasty patients were analysed pre-operatively and for a defined post-operative period to determine the urinary levels of neopterin and total neopterin (neopterin +7,8-dihydroneopterin) using high performance liquid chromatography with fluorescence detection. These results were then compared to a control group of 20 participants with normal knee function.

Results: 7,8-Dihydroneopterin was stable in urine over 12 h when refrigerated. Knee arthritis was associated with an increase in pre-operative neopterin (oxidative stress) and total neopterin (inflammation). The subsequent arthroplasty surgery generated a significant increase neopterin and total neopterin. Both biomarkers were reduced immediately post-operatively, before becoming elevated on the following days. There was no clear evidence of an association between initial neopterin and total neopterin levels and a patient's level of inflammation during in-hospital recovery.

Conclusions: The stability of 7,8-dihydroneopterin in urine allows for its use as an inflammatory marker. Urinary neopterin and total neopterin provided a fast, non-invasive, and simple measure of oxidative stress and inflammation after knee arthroplasty.

1. Introduction

Orthopaedic surgery is a controlled and deliberate trauma to the human body that triggers inflammatory cascades with the production of associated biomarkers. Knee arthroplasty surgery is extremely common and growing in number with almost 8000 performed in New Zealand in 2016 [33] and predictions of up to a 270% increase by 2030 [20]. It is a successful surgery with satisfaction rates from 81% to 89% [4,36,42]. As with all surgery there is the risk of associated complications, with the most common precipitating revision surgery being loosening of components, pain, and prosthetic joint infection (PJI) which has an inflammatory response [12]. Clinical assessment and the incorporation of biomarkers such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) guide the clinician if there is suspicion for atypical inflammation [13,18,43]. However, these biomarkers are from blood or synovial fluid, requiring either venesection or aspiration, which is more invasive than collection of a urine sample. The validation and implementation of another biomarker that is non-invasive and cost-effective could better help clinician's interpret their patient's inflammatory response to arthroplasty surgery and monitor post-operative inflammatory states such as infection.

Neopterin is a small molecule derived from 7,8-dihydroneopterin, a

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monocyte and macrophage cell synthesised antioxidant, produced during times of immune system activation and inflammation. Synthesis occurs when γ-interferon is secreted by T-cells upon recognition of foreign material; such as damaged tissue and pathogens. γ-interferon greatly activates monocytes and macrophages, thus generating large quantities of 7,8-dihydroenopterin [21,40]. Furthermore, activation of these immune cells generates oxidative stress by producing reactive oxygen species, which oxidises 7,8-dihydroneopterin to its stable oxidation product, neopterin [41]. It is thought that the purpose of 7,8-dihydroneopterin is to provide cellular defence against these endogenously generated oxidants [7,14–16]. These ties to the immune system mean neopterin is commonly used as a biomarker when investigating infection from viral or bacterial origins [3]. Serum neopterin was noted to be elevated in patients undergoing elective surgery, indicating that surgery has a biochemically measurable effect on the immune system [9]. However, all the measurements in these studies were made from plasma or serum and do not account for the rapid clearance of neopterin through the renal system [8], where it pools in the urine and can be detected due to its highly fluorescent properties [26].

7,8-Dihydroneopterin does not share neopterins highly fluorescent characteristic. Therefore, it is oxidised into neopterin by acidic iodide to allow detection [10,11]. This measurement is deemed 'total neopterin' and represents neopterin plus 7,8-dihydroneopterin. 7,8-Dihydroneopterin has been shown be labile to varying degrees depending on the solution it is in and the conditions it is exposed to [6]. The stability of urinary 7,8-dihydroneopterin has yet to be investigated and will be analysed in this study at multiple temperatures for further validation of its use as a biomarker.

Urinary neopterin and total neopterin have recently been used to successfully investigate trauma caused during Rugby Union games and Mixed Martial Arts fighting [27,29]. In both of these sports players and fighters receive severe physical trauma causing inflammation, which was measured and quantified using neopterin and total neopterin [27,29]. These markers could be used to measure and investigate an individual's response to trauma in a medical setting, and when combined, give greater elucidation to the balance of oxidative stress and inflammation.

The aim of this study was to biochemically investigate the short-term effects of oxidative stress and inflammation following knee arthroplasty surgery for the purpose of validating the use of neopterin and total neopterin (neopterin plus 7,8-dihydroneopterin) as urinary biomarkers of oxidative stress and inflammation in an orthopaedic clinical setting. Our hypotheses were: 1) That before knee arthroplasty there is an increase in urinary levels of neopterin and total neopterin over controls due to the inflammatory nature of arthritis and 2) that knee arthroplasty surgery will increase the levels of these urinary biomarkers.

2. Methods

2.1. Study design and population

This was an observational cohort study investigating a diagnostic test with Level III evidence. Following local human ethics approval (University of Canterbury Human Ethics Committee HEC 2016/38/LR-PS) and informed consent, 20 control subjects (aged 37.7 ± 12.9, mean ± SD) fulfilling recruitment criteria were selected (Fig. 1). Inclusion criteria for controls required being free from any illness, trauma (including knee disorders), or infections, and not to be taking any medications believed to interact with the immune system in a significant manner. These control subjects produced urine samples each day at midday, for 5 consecutive days. The surgical cohort involved 19 patients aged 62.68 ± 8.97 years (mean ± SD) undergoing elective total knee replacement surgery for arthritis at Burwood Hospital, Christchurch, New Zealand. The difference is age between the control and surgical group was considered to be inconcequential due to the lack of significant difference in neopterin levels in people aged between 18 and 75 [39]. Patients had either a single knee replaced (n = 15), or bilateral total knee replacement (n = 4). Due to a lack of statistical difference in neopterin or total neopterin values between unilateral and bilateral groups, these patients were grouped together as the surgery group. Ethics approval (New Zealand Health and Disability Ethics Committee 16CEN84) and informed consent was gained from the surgical patients. Exclusion criteria were; patients below 18 or above 80 years of age, smokers, or patients having recently received a diagnosis of cancer. Patients provided urine samples before surgery, immediately after surgery, and each day following at approximately midday (± 2 h) until the patients were discharged.

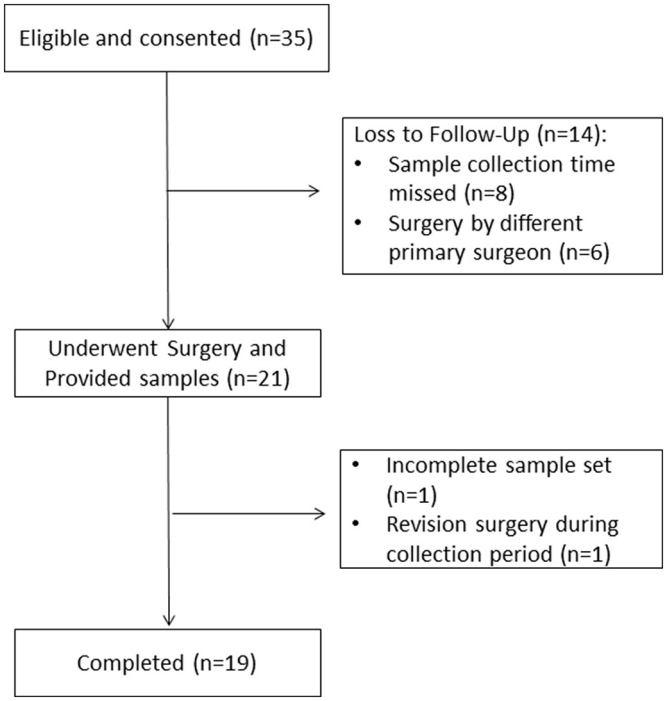

Fig. 1. CONSORT diagram of patient analysis. This diagram illustrates the dropout causes for patients that were eligible and consented for the study.

2.2. Surgery description

Each patient was operated on by the same surgeon (GH) with the same medial parapatellar approach and surgical methodology. All patients had a tourniquet applied proximal to the surgical site and inflated to 350 mmHg. An anterior midline incision was made over the knee with a medial parapatellar dissection and exposure of the joint. Jig-assisted preparation of the distal femur and tibial plateau was performed with subsequent insertion of the appropriate implants. All components were uncemented in each patient and all wounds were drained for 24 h. Each patient received intravenous Tranexamic Acid 1 g following the procedure.

2.3. Anaesthetic description

All anaesthetics were performed as per each anaesthetist's routine practice. Standard medications used to induce and maintain anaesthesia for all patients were Midazolam, Fentanyl, Propofol, Dexamethasone, Rocuronium, Ondansetron, Cyclizine and Paracetamol. At induction all patients were given intravenous Cefazolin 2 g which was continued for a further 24 h post-operatively. Post-operative pain management was obtained using Paracetamol, Codeine and Fentanyl.

2.4. Urinary myoglobin analysis

Urinary myoglobin was measured by HPLC as previously described [26]. Briefly, urine was diluted 5 times in 10 mM ammonium acetate, at pH 7.0 before being injected into the same HPLC setup used to neopterin analysis (below). Separation was achieved using a Phenomenex Jupiter C5 5 μm RP 300 Å1 150 × 4.6 mm column running H_2O + 0.1% (v/v) trifluoroacetic acid, with an acetonitrile gradient up to 70%. Detection of the heme present in myoglobin was made at 400 nm absorbance detection.

2.5. Sample preparation and specific gravity measurements

Urinary sample collection into 70 mL pottles was supervised by the nurses of the orthopaedic recovery wards, before being refrigerated. Samples were taken pre-surgery, as soon as the patient was able post-surgery, and then each day consecutively, at 12 pm, until the patient was discharged (typically 3–4 days). Care was taken to ensure samples were kept under low light conditions and rapidly refrigerated after being produced. These samples were then collected daily and transported on ice to the University of Canterbury before being aliquoted and stored in a − 80 °C freezer.

Urine samples were defrosted in the dark to prevent ultraviolet light mediated degradation of 7,8-dihydroneopterin. 5 μL of urine was vortexed with 195 μL of 20 mM ammonium phosphate (pH 2.5) before 100 μL was transferred into high-performance liquid chromatography (HPLC) vials for neopterin analysis. For total neopterin analysis, 20 μL of acidic iodide was added to the remaining 100 μL of diluted sample, before being vortexed and left in the dark at room temperature, to oxidise the 7,8-dihydroneopterin into neopterin. After 15 min, 10 μL of 0.6 M ascorbic acid was added, followed by vortexing, to quench the acidic iodide reaction. 100 μL of this solution was then transferred into HPLC vials for total neopterin analysis.

HPLC measurements were made using a Shimadzu 20A HPLC with a Sil-20A autosampler with RF-20Axls fluorescence and M20A SPD absorbance detectors. Neopterin separation was achieved with a Phenomenex Luna 5 μm SCX 100 Å 250 mm × 4.6 mm column, using 20 mM ammonium phosphate pH 2.5 as a mobile phase being pumped at 1.0 mL min-1. Fluorescence wavelengths for neopterin detection were set at 438 nm and 353 for emission and excitation, respectively. Peak analysis and result quantification was conducted using Shimadzu Lab Solutions version 5.86.

Specific gravity (SG) was measured to standardise urinary dilution using an ATAGO N-20 refractomter, and calculated as described in [28], using the formula described below and based on the normal population $SG_{1.020}$ [17]. This method is preferred to creatinine due to ease of measurement and reliability [28]. Before measurement, samples were bought to room temperature to remove and temperature-dependent density variation bias [2].

$$[\text{neopterin}](\text{nM}/SG_{1.020}) = (SG_{1.020} - 1)/(SG_{\text{sample}} - 1) \times [\text{neopterin}](\text{nM})$$

2.6. Statistical analysis

Neopterin and total neopterin concentrations and the ratio of neopterin and total neopterin concentrations were compared between the surgery groups and controls using independent *t*-tests and between pre and post-surgery times in the surgery group using paired *t*-tests. A two-tailed p-value < .05 was taken to indicate statistical significance. Neopterin and total neopterin values were $\log_e$ transformed prior to analysis to normalise the distributions. These calculations were made using SPSS version 25. Neopterin and total neopterin values for the stability testing were analysed by two-way ANOVA using GraphPad Prism version 7.0.

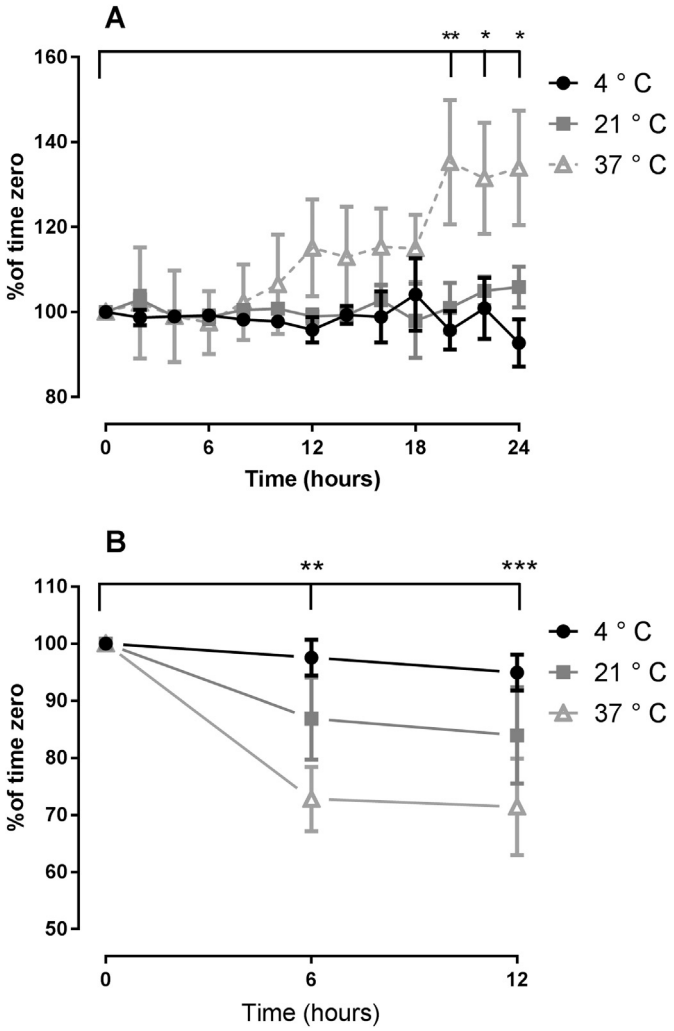

Fig. 2. Stability of urinary neopterin (A) and 7,8-dihydroneopterin (B) at different temperatures. Neopterin was measured by injection of undiluted urine directly into the HPLC system over 24 h (n = 3). 7,8-Dihydroneopterin loss over 12 h (n = 6) was measured by HPLC as neopterin after conversion using acidic iodide oxidation. Data is presented as % change from time 0 with SD. Statistics are significant at the 37 °C condition. *p < .05 **p < .01, ***p < .001.

3. Results

The stability of neopterin and 7,8-dihydroneopterin was examined in urine samples, selected at random by random number generator from the study population. The stability of neopterin in the urine samples was analysed by directly injecting 1 μL of unbuffered urine into the HPLC system. There was no significant change in the neopterin levels at 4 and 21 °C over 24 h, whereas at 37 °C there was a significant increase in neopterin of 34% by 24 h (Fig. 2A). The stability of the 7,8-dihydroneopterin was examined by pre-HPLC oxidation of the 7,8-dihydroneopterin to neopterin by acidic iodide treatment. In urine the 7,8-dihydroneopterin was found to have a high degree of stability over 12 h at 4 °C, with only 5% of the compound being degraded (Fig. 2B). At room temperature (21 °C) this loss is increased to 16% over 12 h. At 31 °C there is statistically significant loss of 7,8-dihydroneopterin at 6 (p < .01) and 12 (p < .001) hours of 27% and 28%, respectively.

Examination of the surgery patients urine showed that neopterin and total neopterin levels were significantly elevated (p ≤ .01 and p ≤ .001, respectively) pre-surgery over healthy controls (Fig. 3). Neopterin levels (Fig. 3A) were significantly higher (p ≤ .05) on day 2 than pre-surgical values. No significant difference was found between

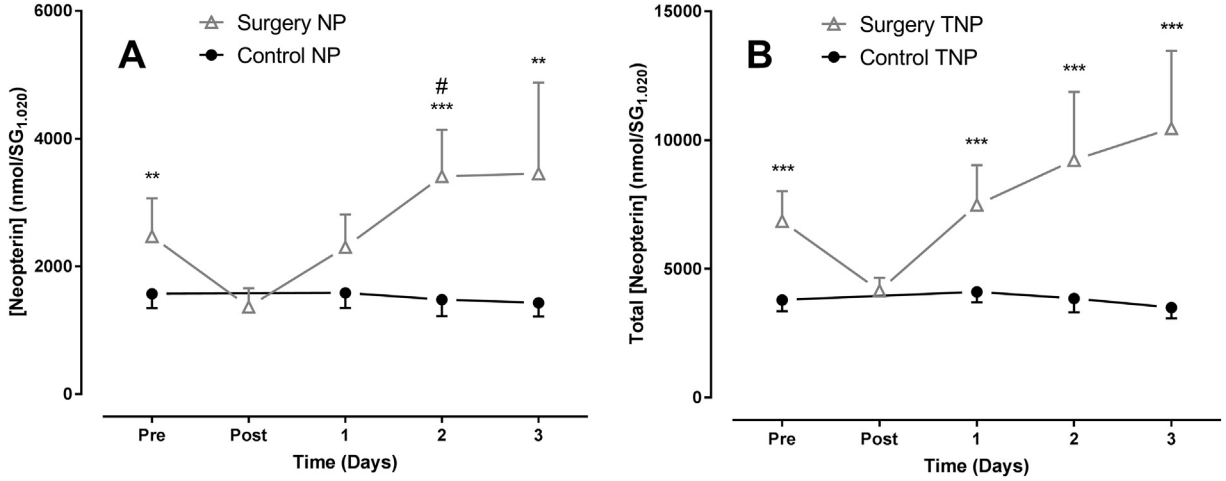

Fig. 3. Urinary neopterin (A) and total neopterin (B) concentrations between healthy controls and knee replacement surgery patients. Knee replacement patient samples were taken pre- and post-operatively, followed by three addition days during in-ward recovery. Healthy control samples were collected for 4 consecutive days. Each data point represents the mean with 95% confidence intervals. Surgical versus controls *p < .05, **p < .01, ***p < .001. Post-operative day 2 versus pre-operative #p < .05,

single and bilateral total knee replacements, resulting in their data being combined. Immediately post-surgery, urinary neopterin and total neopterin levels fall into the control range before becoming elevated back to pre-surgical levels on day 1. No relationship was found between the patients' level of neopterin or total neopterin and anaesthetic drugs administered. None of the patients experienced any serious medical complications or infections which could otherwise alter levels of inflammation during the study period.

To observe dynamics between the cellular inflammatory response and oxidative stress we have calculated the total neopterin/neopterin (TNP/NP) ratio (Fig. 4). This data indicates an increased pre-surgical immune response with a ratio of 2.93 total neopterin per neopterin, versus that of control data (2.51). The change in TNP/NP ratio for the surgical group remains linear over the pre-surgery, post-surgery and day 1 samples. The surgical TNP/NP ratio was largest at day 1 before descending back to pre-surgical range. While these mean changes are not statistically significant (due to the high individual variability in TNP/NP ratios) they may indicate, in conjunction with the total neopterin values, a potential pattern of increased immune activity in the surgical group. However, this result is only suggestive and may be an artefact of the small sample size, thus requiring further investigation to form a worthy conclusion.

The surgical patients were divided into two sub-groups based on initial neopterin and total neopterin median values (Fig. 5). This distinction classified the sub-groups as high initial neopterin or total neopterin (above the median), and low initial neopterin or total neopterin (below the median). Urinary biomarker levels for both high and low initial neopterin and total neopterin subgroups are significantly different pre-surgery (Fig. 5C and D, p ≤ .01). However, any statistical significance between the groups is lost after the surgery. Visually, the difference in sub-groups is shown as individual patient lines in A and B of Fig. 5, which highlights the variation between individual responses to knee arthroplasty surgery.

Urinary myoglobin levels were assessed using reverse phase HPLC. Of the 19 patients analysed, only 2 had measureable levels at the post-operative time point. The first patient had 5.90 μg/mL of urinary myoglobin on day 2. The second patient had 2.84 and 0.4 μg/mL of urinary myoglobin on days 1 and 3, respectively.

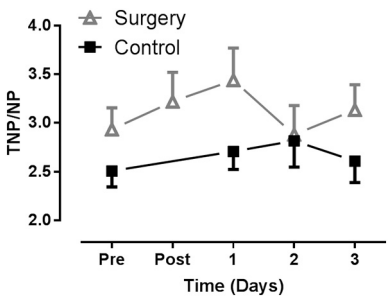

Fig. 4. Urinary total neopterin over neopterin ratios for knee replacement surgery and healthy controls. Knee replacement patient samples were taken pre- and post-operatively, followed by three addition days during in-ward recovery. Healthy control samples were collected for 4 consecutive days. Data is presented as means with SD.

4. Discussion

7,8-Dihydroneopterin was shown to be stable over 12 h when refrigerated at 4 °C. As all samples used for the study were immediately refrigerated at 4 °C and processed within 6 h, we believe that any loss of the compound is negligible and irrelevant to the total neopterin results. The level of neopterin in a sample increases over time (due to the breakdown of 7,8-dihydroneopterin) at 37 °C. This means that oxidation may also be occurring in the bladder, though the bladder environment is low in oxygen, which is required for 7,8-dihydroneopterin oxidation or neopterin [5], so we would expect the oxidation to neopterin to be minimal. The 7,8-dihydroneopterin lost at 37 °C is most likely being converted to neopterin and 7,8-dihydroxanthopterin, as seen in air equilibrated solutions [6]. The data indicates that with minimal care being taken (prompt sample refrigeration), 7,8-dihydroneopterin from urine can be considered a stable biomarker for analysis of monocyte and macrophage activation.

Both urinary neopterin and total neopterin were significantly increased over control levels following knee arthroplasty surgery. The significant elevation in neopterin demonstrates an increased level of oxidative stress occurring before, and increasingly so, after knee arthroplasty. The raised levels of total neopterin reflect an increase in inflammation as 7,8-dihydroneopterin production can only occur upon activation of monocyte and macrophage cells.

Urinary neopterin and total neopterin are both significantly elevated over the control pre-surgery, indicating up-regulation of both reactive oxygen species and monocytes and macrophages, versus that of a healthy person. This supports our first hypothesis and is not unexpected as many knee arthroplasty recipients present with generalised osteoarthritis, which is a chronic inflammatory condition measurable

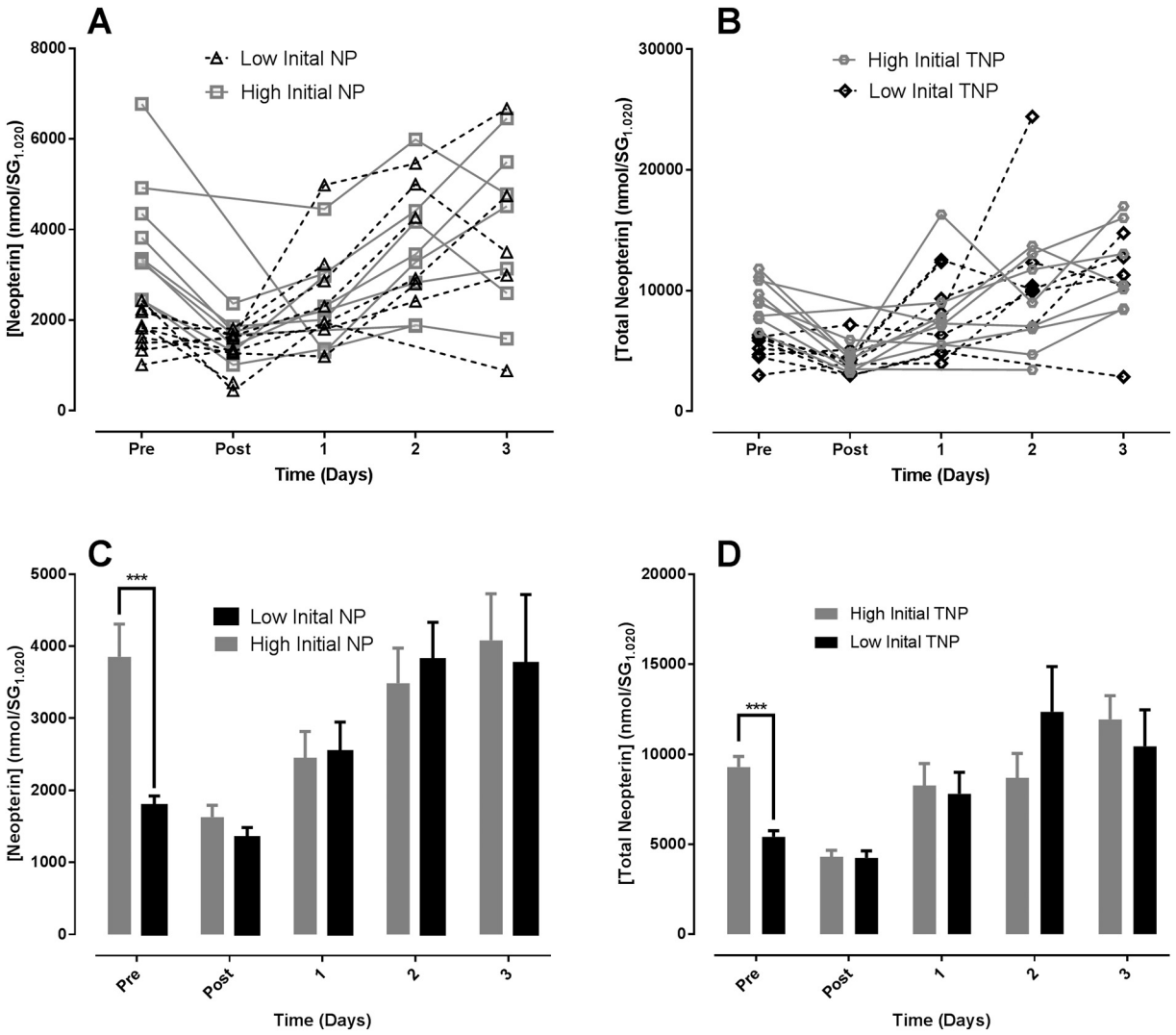

Fig. 5. Individual (A) and grouped (C) neopterin values for high and low initial neopterin (NP) sub-groups and individual (B) and grouped (D) total neopterin for high (grey) and low (black) initial total neopterin (TNP) sub-groups. High and low sub-groups are defined as being above and below the median for initial neopterin and total neopterin values. Grouped data (C, D) are presented as means with SD.

using serum biomarkers [34]. Our second hypothesis is supported by the observation that knee arthroplasty surgery had an up-regulatory effect on both oxidative stress and inflammation, which was reflected in the levels of urinary neopterin and total neopterin. The TNP/NP ratio data suggests that activation of the immune cells may slightly precede the oxidative stress by the ratio increasing slightly over the post- and day 1 values, versus pre-operative values. However, this change is small and not statistically significant; therefore, this is only speculative, with a greater sample size being required for conclusive analysis.

The oxidative stress component of surgical trauma is thought to be, at least in part, due to reactive oxygen species generated upon activation of the immune system, for the purpose of tissue destruction and repair [37]. Our study is the first to investigate the ratio of total neopterin to neopterin in a surgical trauma setting. If oxidative stress and cellular activation were increased equally, we would expect the daily ratio of the two biomarkers to remain constant. However, the data shows that the level of activation of the monocytes and macrophages (total neopterin) grows proportionally larger than the increase of oxidative stress (neopterin) from sterile trauma.

An acute reduction in the levels of urinary neopterin and total neopterin is seen in the immediate post-surgical samples. This decrease in neopterin and total neopterin levels is likely due to the immunosuppression effect of the anaesthetic agents used at surgery, as suggested by previous studies measuring neopterin after cardiac or abdominal surgeries [22,35], and is also seen with other biomarkers used to measure immune system activation ([23,31,32]). The ability to quickly and conveniently measure a patient's level of immune response using urinary neopterin and total neopterin may assist decision making in patient management by clinicians, particularly in cases where immune response can impact patient outcomes. For example in cancer, where decreased immune activity is disadvantageous due to inhibition of natural killer cells [38], or severe immune response syndrome in which the immune system is overactive [24]. The HPLC method used in this work required a 12 min run time. Sample preparation time was minimal, requiring as little as an hour to prepare 50 samples for neopterin and total neopterin analysis. For routine use in a clinical setting this method could be adapted for uHPLC to reduce run times.

The linearity of TNP/NP ratios over pre-surgery, post-surgery and day 1 suggest that the immunosuppression effect alters oxidative stress and immune cell activation equally. This implies that the oxidative stress component of surgery is being generated endogenously from activation of the immune system. The TNP/NP ratios also show heightened levels of inflammation with lower levels of oxidative stress relative to inflammation, compared to the control group. It may be possible that certain inflammatory conditions are selective in which components of immune cells are activated, e.g. high 7,8-

dihydroneopterin production with low production of reactive oxygen species, which has recently been seen in Duchenne muscular dystrophy patients [30]. Reanalysis of the data from a previous publication on the plasma neopterin measurement of ten septicaemia patient showed the ratio of total neopterin to neopterin ratio range of between 1.4 and 1, except for one patient with a ratio of 2.8 [10]. This suggests that in severe infections there is a large and significant oxidation of 7,8-dihydroneopterin to neopterin. Further clinical studies and complementary in vitro work would be required to explore this mechanism.

Individual data is presented in Fig. 5A and B to demonstrate the variance amongst the population of surgical patients. From these data, high and low initial neopterin and total neopterin subgroups were also characterised and presented. While the initial pre-surgical values are significantly different as expected, this difference is not maintained for the three days following surgery. Therefore, initial neopterin and total neopterin levels may not be suitable as short-term predictors of an individual's inflammatory response following knee arthroplasty. However, given that neopterin has shown merit in other studies as a pre-surgical predictor of sepsis and organ failure following major trauma [1,19], it may be that initial levels of these markers could predict a more long term recovery outcome. Further work is required to investigate this.

Unlike physical contact sports which cause a high degree of muscle damage [26,27], knee arthroplasty does not lead to significantly elevated levels of urinary myoglobin, indicating considerably lower levels of muscle trauma. As serum myoglobin has been detected after knee arthroplasty [25], our result was thought to be due to very low levels of muscle damage combined with the flux of myoglobin into urine causing a dilution effect that puts urinary myoglobin levels below the detection limits of the analysis method (detection of heme by absorbance reverse phase HPLC).

This study has shown it is possible to measure a patients level of oxidative stress and inflammation using non-invasive biomarkers. Future research would aim to investigate and compare the changes in urinary neopterin and total neopterin over an extended period of time after knee arthroplasty, with comparisons to a more invasive and traumatic surgery. The lack of statistically significant difference between single and bilateral knee arthroplasty is likely due to the low n value (4) for the bilateral group. Due to this lack of statistical significance the results have been interpreted as a single group. Larger n values and an extended sampling time course over several weeks may demonstrate a difference in inflammatory biomarkers and recovery between unilateral and bilateral knee arthroplasty due to the difference in the level of trauma. Extended sampling may also allow for case studies in the event that PJI occurs, and help investigate any potential for initial neopterin and total neopterin values to be a predictor of long term recovery.

5. Conclusion

Urinary neopterin and total neopterin are stable, reliable and informative biomarkers when applied to knee arthroplasty surgery. Both biomarkers are elevated prior to surgery and become further elevated after surgical trauma. The dynamics of the increases in neopterin and total neopterin suggests activation of the immune system followed by an increase in oxidative stress. While initial neopterin and total neopterin values could not be used to predict a patients “in-hospital” recovery, it is possible that these values may be of more importance for long term recovery prediction – which is currently being examined by an extension of this study. With urinary biomarkers, sample collection is non-invasive and does not inflect any additional stress on the patient. Analysis is straight forward and rapid and provides another tool in a clinician's armament to monitor inflammation.

Geological information

The present study was conducted in Christchurch, New Zealand. All patients from the surgical cohort were operated on and cared for in Burwood Hospital, Christchurch, New Zealand. The control cohort was residents of Christchurch, New Zealand. All sample analysis was carried out at the University of Canterbury, Christchurch, New Zealand.

Disclosure statement

The authors declare that they have no conflict of interest.

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Author contribution statement

All the credited authors read, appraised and approved the final submitted manuscript. Gregory Baxter-Parker: contributed to study conception, design, technical data acquisition with analysis and manuscript writing. Lloyd Roffe: contributed to clinical data acquisition with analysis and manuscript writing. Steven Gieseg: contributed to study conception, design, technical data acquisition with analysis and manuscript writing. Gary Hooper: contributed to study conception, design and manuscript writing.

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