The Knee 26 (2019) 914–922

Contents lists available at ScienceDirect

The Knee

Stiffness post-total knee replacement: A proof of principle study investigating the effect of gene expression analysis of markers of fibrosis

Charles J. Mann [a,b,*], Anne M. Bradley [a], Eleanor R. Jones [c], Iain R. McNamara [a,b], Toby O. Smith [d], Graham P. Riley [c]

[a] Department of Trauma and Orthopaedics Norfolk and Norwich University NHS Foundation Trust, Colney Lane, Norwich NR4 7UY, United Kingdom of Great Britain and Northern Ireland
[b] Faculty of Medicine and Health Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, United Kingdom of Great Britain and Northern Ireland
[c] School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, United Kingdom of Great Britain and Northern Ireland
[d] Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Botnar Research Centre, Oxford OX3 7LD, United Kingdom of Great Britain and Northern Ireland

ARTICLE INFO

Article history:
Received 26 February 2019
Received in revised form 12 April 2019
Accepted 6 May 2019

Keywords:
Knee arthroplasty
Limited range
Arthrofibrosis
Gene expression
Case–control

ABSTRACT

Background: To establish proof of principle of a link between phenotypic expression and stiffness after TKR.
Methods: From 100 patients, genetic expression of markers of fibrosis was performed for 15 synovial samples from patients categorised as 'best post-operative range of movement (ROM)' and 15 samples from patients with 'worst ROM'. These markers included Matrix Metalloproteinases (MMPs), A Disintegrin and Metalloproteinases with Thrombospondin (ADAMTS) and Tissue Inhibitors of Matrix Metalloproteinases (TIMPs). Genetic marker data were compared to Oxford Knee Scores (OKS) and Pain Catastrophizing Scores (PCS).
Results: Quantitative markers for gene expression demonstrated more outliers in stiff compared to non-stiff knees, suggesting a greater imbalance in pro- and anti-fibrotic markers in stiff knees. Whilst there was a significant difference in the range of post-operative knee flexion ($p = 0.001$) and extension ($p = 0.001$), there was no statistically significant difference between stiff and non-stiff knees in pre-operative or post-operative OKS ($p \geq 0.06$). There was no difference in the individual components of the individual PCS score items nor the PCS total scores when stiff and non-stiff knees were compared ($p > 0.05$).
Conclusion: Biological factors, namely gene expression of MMPs, TIMPs and ADAMTS, may contribute towards post-TKR stiffness. This now warrants further investigation to better understand this relationship based on larger, multi-centre, cohorts.
Level of evidence: Level 3.

1. Introduction

Knee stiffness after Total Knee Replacement (TKR) is not uncommon and can be debilitating [1–3]. Whilst a functional range of motion (ROM) has been defined as 67° of flexion during the swing phase of gait [4], a range less than 90° of flexion can impair stair ascent and

* Corresponding author at: Department of Trauma and Orthopaedics Norfolk and Norwich University NHS Foundation Trust, Colney Lane, Norwich NR4 7UY, United Kingdom of Great Britain and Northern Ireland.
E-mail address: charles.mann@nnuh.nhs.uk. (C.J. Mann).

https://doi.org/10.1016/j.knee.2019.05.005
0968-0160/

rising from a seated position [5]. Patient expectation is now that a minimum of 90° of flexion is achieved post-operatively, and preferably more [6]. Joint stiffness is just one of several, possibly interlinked, reasons why patients are dissatisfied after TKR [7,8].

Factors associated with stiff (and painful) TKRs include component mal-sizing and mal-orientation and poor soft-tissue balancing [9]. However, there are patients who are stiff who do not have an implant or other technical issue. Patient factors such as genetic makeup and pain perception have been implicated as possible causes for knee stiffness post-TKR, but neither factor has been firmly established [10,11].

Disentangling the aetiology of significant post-operative pain and/or stiffness after TKR is difficult [1,2]. Surgical, psychosocial, genetic and central (nervous system) factors may all play a role [13]. From a genetic perspective, there are a number of key genetic markers of fibrosis which may be associated with stiffness post-TKR. Matrix Metalloproteinases (MMPs) have traditionally been thought to degrade extracellular matrix and therefore excessive fibrosis was thought to correlate with a lack of MMP production. However, this may be too simplistic an approach and that wound healing and scar formation are a complex interaction between pro- and anti-fibrotic mechanisms, with MMPs being both pro- and antifibrotic [1,4]. A Disintegrin and Metalloproteinase (ADAM) and A Disintegrin and Metalloproteinase with Thrombospondin Motif (ADAMTS) are active proteases with physiological function similar to MMPs. Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) are functionally reciprocal to MMPs. An imbalance between MMP and TIMP expression would lead to a pro-fibrotic state if the activity of TIMPs was favoured over that of MMPs.

Given this uncertainty in the TKR population, the purpose of this study was to establish proof of principle as to whether there is a link between phenotypic expression and stiffness after TKR and patient-reported pain and disability scores. This is important as determining people at risk of stiffness post-TKR based on genetic markers or symptom presentation could inform targeted treatment both in rehabilitation and potentially pre-operative counselling.

2. Materials and methods

We collected data on 150 consecutive patients who underwent primary TKR at a United Kingdom National Health Service (NHS) university hospital. We included only those patients who had a primary TKR for tibio-femoral arthritis and who had a cruciate retaining Genesis II (Smith and Nephew®, Memphis USA) TKR. Any patient with an inflammatory arthropathy or primary patellofemoral arthritis was excluded in addition to those who required an additional procedure during TKR such as a lateral release, bone grafting or implant augment. We also excluded any patient who needed a significant further distal femoral resection of greater than two millimetres (additional to the standard primary distal femoral cut) or who required a polyethylene insert of greater than 15 mm. Valgus knees were not excluded unless this was severe enough to warrant a posterior stabilized implant.

All patients followed a standard post-operative recovery programme including early mobilisation Day 1 post-operatively and post-operative physiotherapy and occupational therapy prior to discharge. All patients were discharged with a home exercise plan and reviewed by their surgical team at six weeks post-operatively.

2.1. Tissue sample and gene expression analysis

Synovial tissue for gene expression analysis was collected intra-operatively from the supra-patella pouch. This tissue was chosen as it is routinely sacrificed to accurately measure the size of the femoral component, and fibrosis in the supra-patella pouch is known to be associated with post-operative adhesions and stiffness post-TKR [10,14]. This tissue was immediately placed after collection into a solution of RNAlater at four degrees Celsius for 24 h and subsequently frozen at −80 °C until the tissue was required for analysis.

Samples from 100 patients were obtained. For all patients who donated samples, knee flexion (mean: 106°) and extension ROM (mean: −2°) was measured both pre-operatively and at six-weeks post-operatively. Based on this, 15 patients were categorised into a 'best ROM post-operatively' subgroup and 15 in a 'worst ROM post-operatively' subgroup, representing the highest or lowest values from within the cohort respectively. This sample size was determined based on previous recommendations of required proof of principle sample size [10]. The ROM for these subgroups is presented in Tables 1 and 2. The samples for these individuals underwent gene expression testing and were analysed using reverse transcription and qRT-PCR. In this process, RNA was extracted using the ultraturax followed by the tri-spin RNA extraction protocol [15]. Tissue was immersed in trizol (one millilitre per 100 mg of tissue) and homogenised using the ultraturax (2 × 10 second blasts). One hundred twenty five microgrammes per litre of glycogen was added and incubated at room temperature for three minutes. Chloroform with a sample volume of 2/5 was added to the samples

Table 1
Demographic characteristics for the stiff and non-stiff knee groups.

	Stiff knee group	Non-stiff knee group	Difference (p-value; 95% CI)
N	15	15	
Mean age (SD)	64.93 (8.40)	68.47 (9.56)	0.291 (−10.26 to 3.20)
Gender (m/f)	2/13	7/8	0.919 (not estimateable)
Mean pre-operative extension (SD)	−6.47 (6.16)	−3.87 (4.98)	0.214 (−6.79 to 1.59)
Mean pre-operative flexion (SD)	95.00 (9.78)	95.53 (15.97)	0.913 (−9.37 to 10.44)
Mean pre-operative OKS (SD)	17.73 (5.48)	15.80 (7.88)	0.307 (−2.46 to 7.52)

CI: confidence interval; f: female; m: male; OKS: Oxford Knee Score; SD: standard deviation.

Table 2
Post-operative comparisons for Oxford Knee Score and Pain Catastrophizing Scale score for the stiff and non-stiff knee groups.

	Stiff knee group	Non-stiff knee group	Difference (p-value; 95% CI)
N	15	15	
Mean post-operative extension (SD)	−10.07 (7.33)	−2.07 (2.37)	0.001 (3.92 to 12.08)
Mean post-operative flexion (SD)	78.00 (13.34)	105.67 (6.78)	0.001 (−35.57 to −19.75)
Mean post-operative OKS (SD)	23.46 (8.62)	21.47 (7.18)	0.06 (−10.72 to 0.19)
Mean post-operative PCS (Total Score) (SD)	18.60 (10.52)	19.00 (16.60)	0.938 (−9.99 to 10.79)
Mean post-operative PCS (Rumination) (SD)	6.73 (5.27)	6.33 (6.00)	0.848 (−4.62 to 3.82)
Mean post-operative PCS (Magnification) (SD)	2.87 (1.41)	4.00 (3.68)	0.275 (−0.95 to 3.22)
Mean post-operative PCS (Helplessness) (SD)	9.00 (5.32)	8.73 (7.57)	0.912 (−5.16 to 4.63)

CI: confidence interval; f: female; m: male; PCS: Pain Catastrophizing Scale Score; OKS: Oxford Knee Score; SD: standard deviation.

which were shaken vigorously for 15 s then incubated at room temperature for five minutes. After centrifugation (at 12,000 ×*g* for 15 min) the upper phase was transferred into a fresh 1.5 ml tube. An equal volume of isopropanol was added, and samples were incubated at room temperature for at least 10 min. Samples were centrifuged for 10 min at 12,000 ×*g* and the supernatant was discarded. Pellets were washed with one millilitre of ethanol (vortexed then centrifuged for five minutes at 7500 ×*g* before the ethanol was removed), air dried and re-suspended in 50 μl of analytical grade water.

RNA was reverse transcribed into a cDNA library using the superscript II kit from Invitrogen® (Carlsbad, CA. USA) as per the manufacturer's instructions. RNA was primed with random hexamers and reverse transcribed with the superscript II kit according to manufacturer's instructions. In brief, RNA was incubated at 70 °C for 10 min with 200 ng random hexamers. Four microlitres of 5× sample buffer, 10 mM of DTT, 0.125 mM of dNTPs (2.5 mM), 200 units of Superscript II and 40 units of RNase inhibitor were added and incubated for one hour at 42 °C and at 70 °C for 10 min. To determine whether the reverse transcription had worked effectively, standard Taqman© (Thermo Fisher Scientific, MA, USA) analysis of the house-keeping gene was performed on a sample of the cDNA.

The standard qRT-PCR programme was run using the Applied Biosystems 7500 real time PCR system. Each reaction was performed in a volume of 25 μl including; 10 ng cDNA, 33% KAPA Probe fast qPCR kit Mastermix (2×), 0.2 nM each of the forward and reverse primer and 0.1 nM of probe. The thermal cycles were as follows: 50 °C for two minutes, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for one minute. Following this, Taqman low density array was performed on the samples. Five hundred nanogrammes of cDNA was loaded onto each port of custom designed 48 gene/port TLDA array card and run according to the manufacturer's instructions using the Applied Biosystems 7900HT Real-Time PCR System and Applied Biosystems Sequence Detection Systems (SDS 2.3 and RQ manager 1.2) software (Thermo Fisher Scientific). The thermal cycles were as follows: 50 °C for two minutes, 95 °C for 10 min followed by 40 cycles of 97 °C for 30 s and 60 °C for one minute. Data were normalised to the most stable housekeeping gene, beta actin, and expressed as 2^−Delta Ct.

The following broad groups of genes were tested: Extracellular Matrix Metalloproteinases (ADAMTS), Matrix Metalloproteinases (MMPs) and inhibitors of MMPs (TIMPs). A full list of the genes tested is shown in Supplementary Table 1.

2.2. Clinical assessment

We collected pre-operative and six-week post-operative Oxford Knee Scores (OKS) [16] and Pain Catastrophizing Scale scores (PCS) [17]. The Oxford Knee Score is a 12-item, participant-completed questionnaire which has demonstrated reliability and validity for the assessment of knee disability for the TKR population [17]. The PCS is a 13-item participant completed questionnaire which is reliable and valid for the assessment of perceived pain experience in people with chronic pain [19]. It comprises three sub-sections (rumination, magnification, helplessness) to form a total score. We also collected pre-operative and six-week post-operative knee flexion and extension ROM measured by a single researcher (AB) following a standardised procedure using a goniometer [20]. This was performed with the participant seated at the edge of the examination couch and asked to actively flex their knee as far as possible. The bony landmarks of the lateral malleolus and the greater trochanter of the hip were identified and the axis of the goniometer was placed on the lateral aspect of the knee joint line.

We defined knee stiffness as either a failure to achieve 90° of flexion and/or a total ROM of less than 90° at six weeks post-operatively.

The six week post-operative x-rays of those patients who were tested for genetic expression were examined by a consultant knee surgeon (IM) to exclude surgical causes of stiffness. Post-operative plain x-ray films were examined using the institute's Picture Archiving and Communication System (PACS), for alignment, over or under-sizing, and overstuffing of the patellofemoral joint. The examiner was blinded as to whether the patient was stiff or not.

2.3. Data analysis

Descriptive statistics were used (mean and standard deviation) to assess the difference in genetic expression (ADAMTS, MMPs, TIMPs) between 15 participants who presented with post-operative stiff knee, and the 15 participants who did not have a stiff knee. As this is a proof of principle study with only 30 participants, inferential statistical tests were not performed to assess genetic expression. However within-group pre- versus post-operative differences for OKS and PCS were assessed using the non-parametric tests the Wilcoxon Matched Pairs Test, whilst the Mann–Whitney Test was used to assess the between-group

pre- versus post-operative differences for OKS and PCS. Statistical significant was denoted as $p < 0.05$. All statistical analyses were performed on SPSS version 21.0 (IBM, New York, USA).

3. Results

3.1. Cohort characteristics

The characteristics of the 30 participants who underwent gene expression analysis are presented in Table 1. As this illustrates, there were no statistically or clinically significant differences between the groups in respect to characteristics

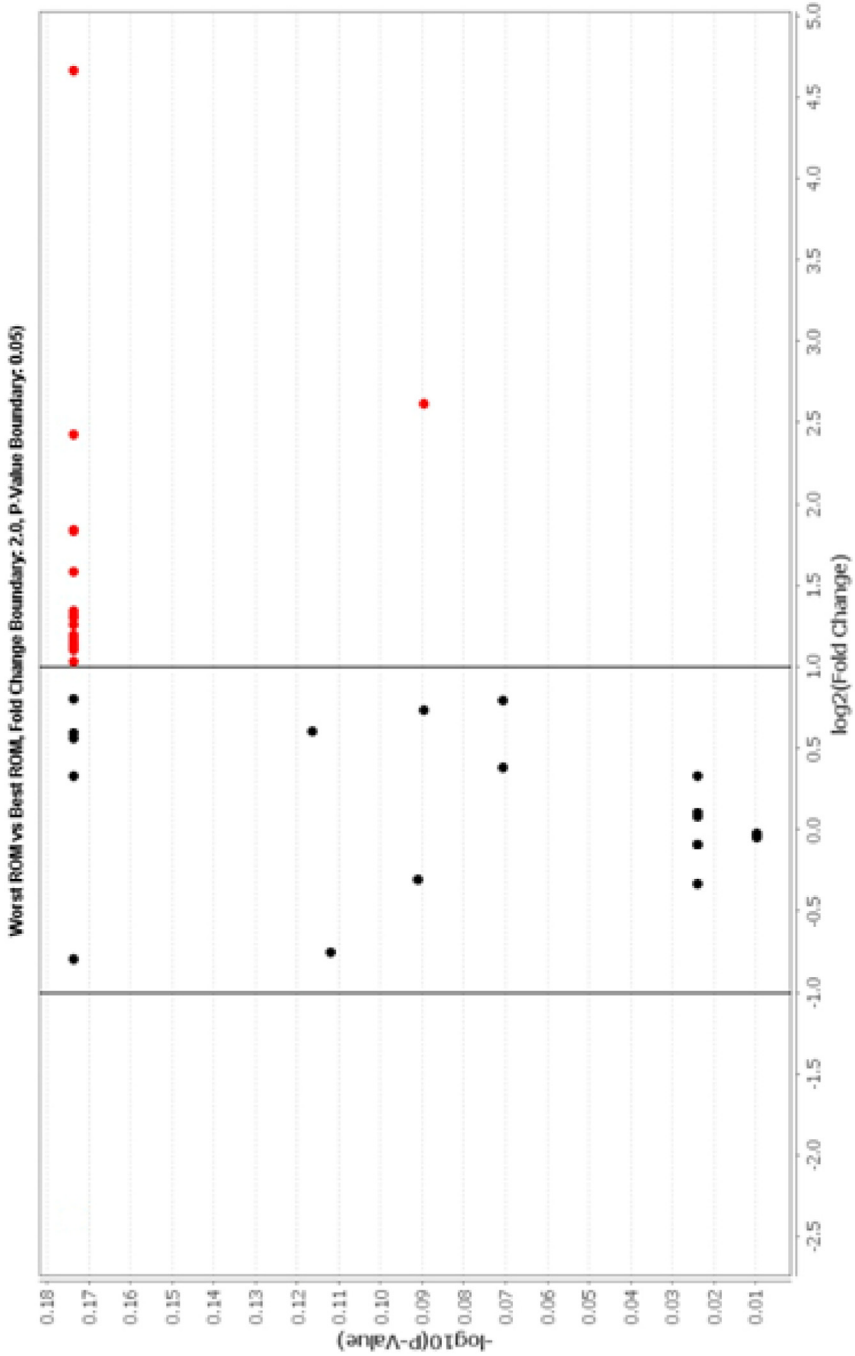

Figure 1. Volcano plot comparing best versus worst knee range of movement.

with the exception of a greater proportion of the stiff knee cohort being female compared to the non-stiff knee cohort (86.7% vs. 53.3%).

3.2. Gene expression analysis

Figure 1 illustrates a trend for an overall difference between the stiff and non-stiff knees for fibrotic characteristics. These are confirmed by the individual gene analyses (Figures 2–4) indicating a trend for a difference in gene expression findings between the two cohorts. There was greater variance in gene expression in the stiff group. There was consistently a higher mean value for MMPs, TIMPs and ADAMTS for the stiff group compared to non-stiff group (Figures 2–4).

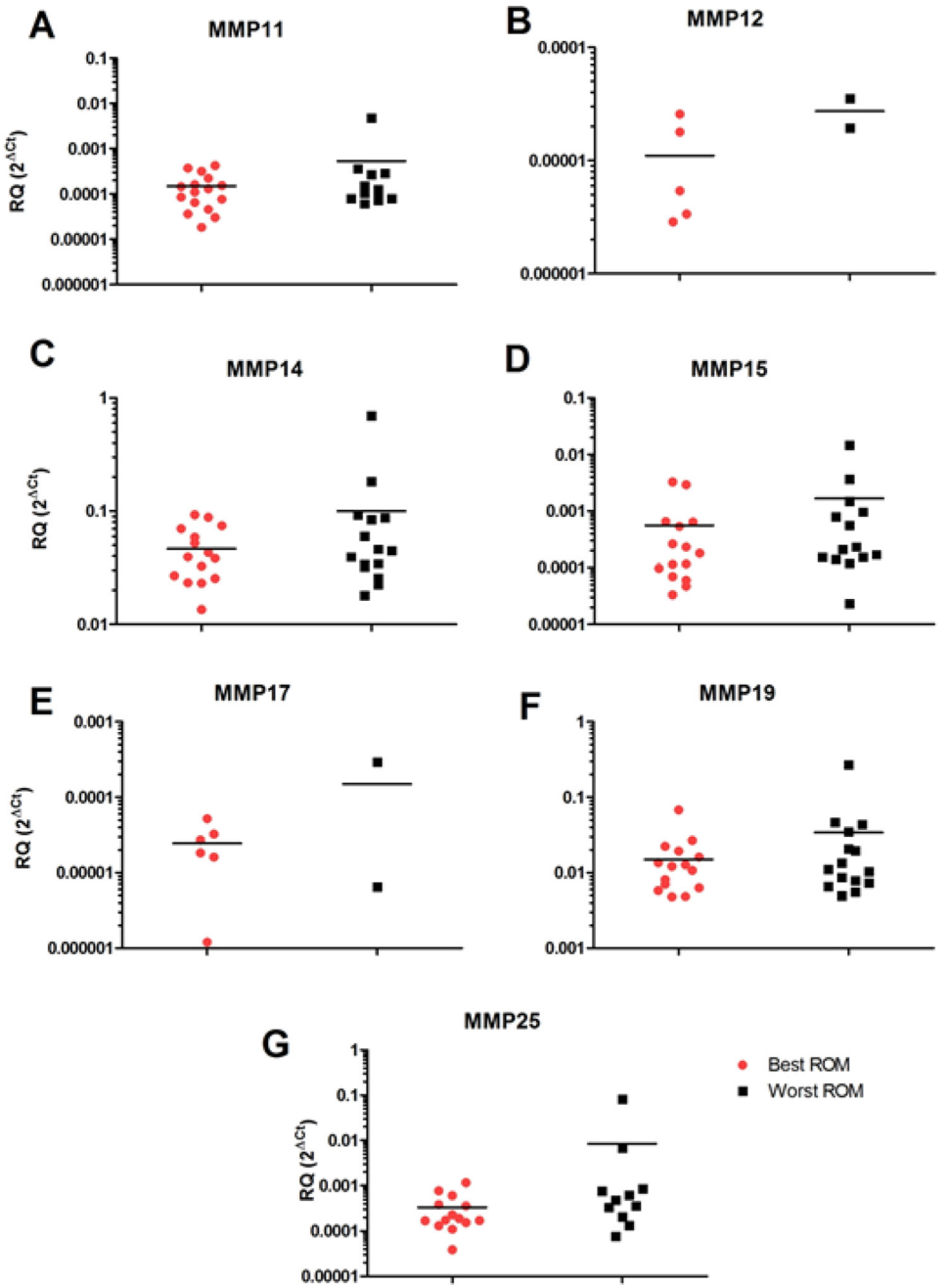

Figure 2. MMP gene expression in synovial samples divided into worst and best range of motion.

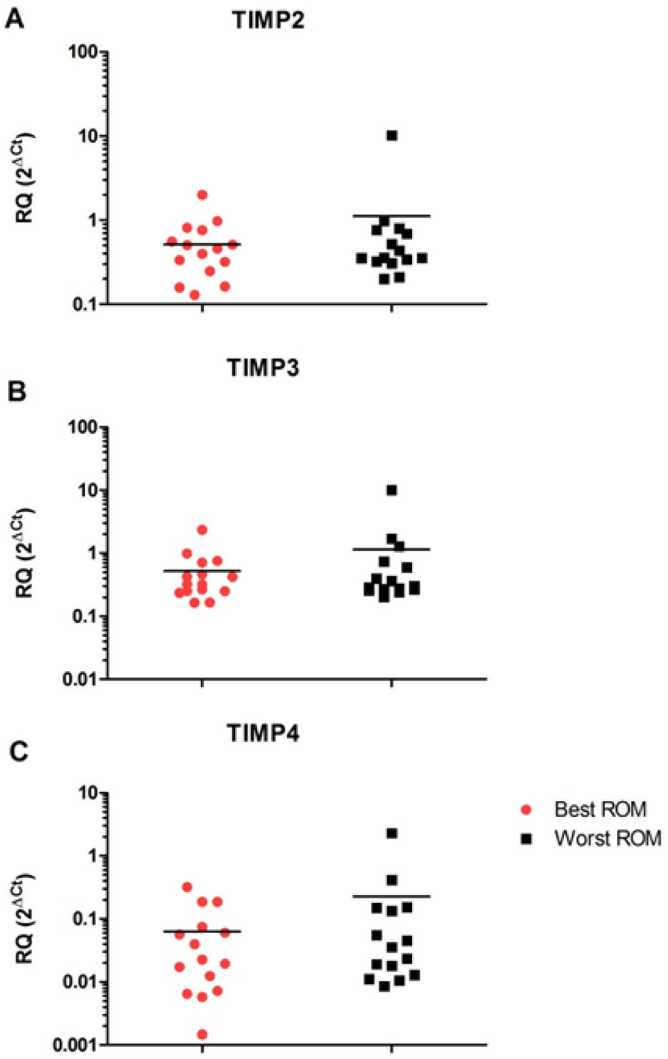

Figure 3. TIMP gene expression in synovial samples divided into worst and best range of motion.

3.3. X-ray analysis

There were no significant differences in component alignment, size or overstuffing between the two groups. There was one set of x-rays missing for one patient who was in the non-stiff group. There were two patients whose overall x-ray appearance was satisfactory but had mild overstuffing of the patellofemoral joint. These were in the non-stiff group.

3.4. Clinical analysis

The results for analysis comparing pre-operative and post-operative outcomes between the stiff and non-stiff knee groups are presented in Tables 1 and 2. There was no clinically or statistically significant difference between the stiff knee and non-stiff knee groups for pre-operative total OKS (mean: 17.7 vs. 15.8; $p = 0.31$) or post-operative score (mean: 23.5 vs. 21.5; $p = 0.06$). There was no significant difference between the change in OKS pre- to post-operatively between the stiff and non-stiff knee groups (mean: 7.2 vs. 13.7; $p = 0.14$).

There was no clinically or statistically significant difference for total PCS score (mean: 18.6 vs. 19.0; $p = 0.94$). Similarly, there was no difference between the stiff knee and non-stiff knee groups post-operatively for the specific sub-domains of the PCS (Rumination, Magnification and Helplessness) ($p \geq 0.28$; Table 2).

Whilst there was no statistically significant difference in pre-operative range of knee extension or flexion ($p \geq 0.31$; Table 1), post-operatively there was a difference in both knee extension ROM (mean: −10.1 vs. 2.1; $p = 0.001$) and flexion ROM (mean: 78.0 vs. 105.7; $p = 0.001$).

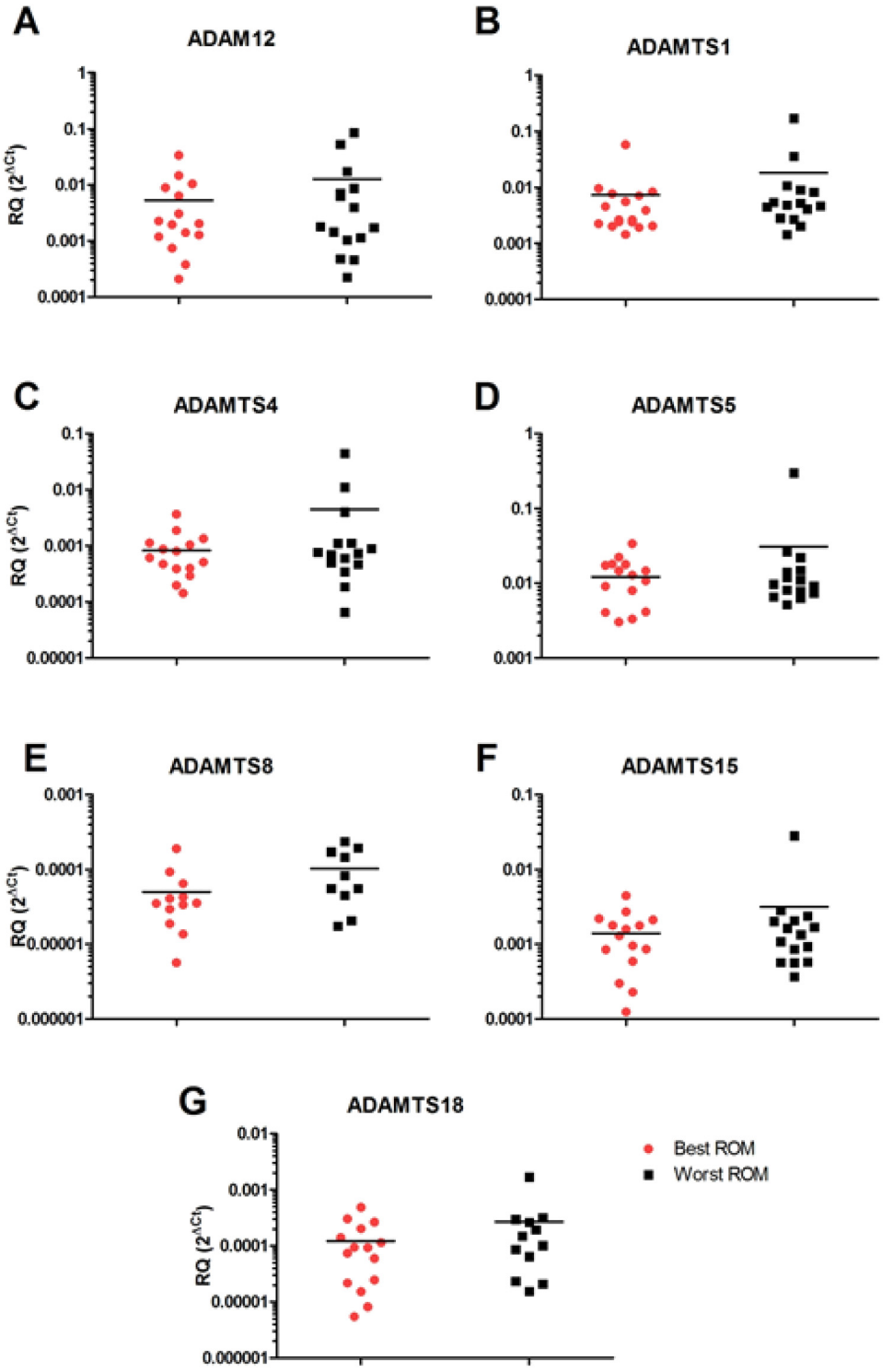

Figure 4. ADAM and ADAMTS gene expression in synovial samples divided into worst and best range of motion.

4. Discussion

The findings of this study suggest that biological factors may be associated with knee stiffness. There are higher mean values for MMPs, TIMPs and ADAMTS in stiff knees compared to non-stiff knees. However, based on this cohort, there is limited evidence

of a relationship between knee stiffness and PCS. The results are based on a small number of cases. Further study is warranted to explore whether this trend in finding remains true when tested with a larger, multi-centre, cohort.

There is a subtle balance between enzyme activity which is essential for wound healing and that which may promote excessive fibrosis which may be associated with post-operative joint stiffness [21]. MMP activity is essential during the proliferative phase of wound healing as the wound is 'rebuilt'. MMP is also necessary for the remodelling or maturation phase [22]. This is a desirable effect as far as surgical incision wound healing by primary intention is concerned, but is deleterious as far as wound healing by secondary intention is concerned as there is a large raw area of exposed tissue when a TKR is performed [21]. The myofibroblastic activity reaches a peak at around 15 days and is much reduced by 30 days after the wound was created [21]. Thus the balance between MMP activity and TIMP activity is crucial [21,22]. Our results concur with this suggesting that ADAMTS and MMP activity is not significantly counteracted by TIMP in the stiff knee patients.

Our study did not demonstrate a significant difference in PCS scores between the stiff and non-stiff groups, even when the individual subsections contributing to the total score were analysed. This may be attributed to the small number of patients analysed, thereby being influenced or the tool being insensitive to detect a change in this population. Other tools may be more appropriate, such as those which assess global pain and psychological distress, such as fibromyalgia scoring systems [22] compared to the PCS score. Whilst neither tool is designed to distinguish between individuals who may do more poorly because of pain compared to stiffness. However, our results would indicate that stiffness is a significant factor in a functionally poor TKR and should therefore be considered an important but separate domain to evaluate in this subgroup of the TKR population.

This study presented with two principal limitations. First, although radiological assessment was undertaken to assess malrotation through plain x-ray, this may have been more accurately assessed through Computed Axial Tomography scanning. This was not performed as it was considered unethical to subject a normal ROM participant to excessive radiation. Second, although the number of participants was small, it was regarded that this sample size was acceptable to assess proof of principle for genetic expression and knee stiffness [10]. There were no previous papers looking at fibrosis in TKR to estimate a power calculation on. The authors accept that the paper does not provide a definitive answer to a complex problem, but would conclude that further investigation and debate is warranted based on our results.

5. Conclusion

Joint stiffness is a multifactorial problem after TKR but biological factors may play a significant role in stiffness after TKR. The interplay between biological factors and central factors (such as pain perception) may be important but their relationship remains unclear, warranting further investigation. Future definitive investigation would improve understanding on the phenotype of patients who experience stiffness post-TKR, to improve stratified pre- and post-operative care and counselling.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.knee.2019.05.005.

Funding

This research received a research grant from the Gwen Fish Fund (Norfolk, UK) to support the conduct of this study. TOS is supported by funding from the National Institute for Health Research (NIHR) Oxford Health Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the NIHR.

Ethical approvals

Research biobank approval was gained for tissue samples from the University of East Anglia-Norfolk and Norwich University Hospital.

Declaration of Competing Interest

None.

Acknowledgements

None.

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