THE KNEE

TITLE PAGE

Informative Title: Stiffness post-total knee replacement: a proof of principle study investigating the effect of gene expression analysis of markers of fibrosis.

Concise Title: Gene expression of stiffness post-TKR

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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 were 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 ($p\geq0.06$). 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.

KEYWORDS: knee arthroplasty; limited range; arthrofibrosis; gene expression; case-control

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 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]. 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-TKA, 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 factors, psychosocial factors, genetic factors 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-TKA. 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 and that MMPs can be both pro- and antifibrotic [1,4]. A Disintegrin and Metalloprotease (ADAM) and A Disintegrin and Metalloprotease with Thrombospondin Motif (ADAMTS) are active proteases with physiological function similar to MMPs. Tissue Inhibitors of Matrix Metallo Proteinases (TIMPS) are functionally reciprocal to MMPs. An imbalance between MMPs and TIMPS 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-TKA based on genetic markers or symptom presentation could inform targeted treatment both in rehabilitation and potentially pre-operative counselling.

MATERIALS AND METHODS

We collected data on 150 consecutive patients who underwent primary TKR at an 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 patello-femoral 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 2mm (additional to the standard primary distal femoral cut) or who required a polyethylene insert of greater than 15mm. 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.

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 4°C for 24 hours 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: 105.6°) and and extension ROM (mean: -2°) was measured both pre-operatively and at six-weeks post-operatively. Based on this, 15 patients were catagorised 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 are presented in Table 1 and Table 2. The samples for these individuals underwent gene expression testing and 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 (1ml per 100mg of tissue) and homogenised using the ultraturax (2 x 10 second blasts). 125 μg/ml glycogen was added and incubated at room temperature for three minutes. 2/5 sample volume of chloroform was added to the samples which were shaken vigorously for 15 seconds then incubated at room temperature for five minutes. After centrifugation (at 12000 x g for 15 minutes) the upper phase was transferred into a fresh 1.5ml tube. An equal volume of isopropanol was added, and samples were incubated at room temperature for at least 10 minutes. Samples were centrifuged for 10 minute at 12000 x g and the supernatant was discarded. Pellets were washed with 1ml ethanol (vortexed then centrifuged for five minutes at 7500 x g before the ethanol was removed), air dried and re-suspended in 50μl 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 minutes with 200ng random hexamers. 4μl 5x sample buffer, 10mM DTT, 0.125mM dNTP's (2.5mM), 200units Superscript II and 40units RNase inhibitor were added and incubated for one hour at 42°C and at 70 °C for 10 minutes. To determine whether the reverse transcription had worked effectively, standard Taqman©(Thermo Fisher Scientific, MA, USA) analysis of the housekeeping 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 25μl including; 10ng cDNA, 33% KAPA Probe fast qPCR kit Mastermix (2x), 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 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for one minute. Following this, Taqman low density array was performed on the samples. 500ng of cDNA was loaded onto each port of custom designed 48 gene/port TLDA array card and run according to 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, 94.5°C for 10 minutes followed by 40 cycles of 97°C for 30 seconds and 59.7°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.

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 of 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 sat 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 place 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.

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. Given that 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 Wilcoxin Matched Pairs Test, whilst the Mann—Whitney Test was used to assess the within-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).

RESULTS

Cohort Characteristics

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

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 analysis (Figure 2; Figure 3; Figure 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 (Figure 2; Figure 3; Figure 4).

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.

Clinical Analysis

The results for analysis comparing pre-operative and post-operative outcomes between the stiff and non-stiff knee groups are presented in Table 1 and Table 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 group 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).

DISCUSSION

The findings of this study suggest that biological factors may be associated with knee stiffness. There are higher mean value 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 group, 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 mal-rotation 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 the 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.

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.

DECLARATIONS

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Conflict of Interest: None.

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

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FIGURE AND TABLE LEGENDS

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

Figure 2. MMP gene expression in synovial samples divided into worst and best ROM. Data was normalised to β actin and expressed as relative quantification (RQ) 2^-ΔCt. Mean values are indicated by the black lines.

Figure 3. TIMP gene expression in synovial samples divided into worst and best ROM. Data was normalised to β actin and expressed as relative quantification (RQ) 2^-ΔCt. Mean values are indicated by the black lines.

Figure 4. ADAM and ADAMTS gene expression in synovial samples divided into worst and best ROM. Data was normalised to β actin and expressed as relative quantification (RQ) 2^-ΔCt. Mean values are indicated by the black lines.

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

Table 2: Post-operative comparisons for Oxford Knee Score and PCS score for the stiff and non-stiff knee groups.

Supplementary Table 1. Full list of genes tested

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Figure 1: Volcano plot showing fold change boundary for best and worst range of motion

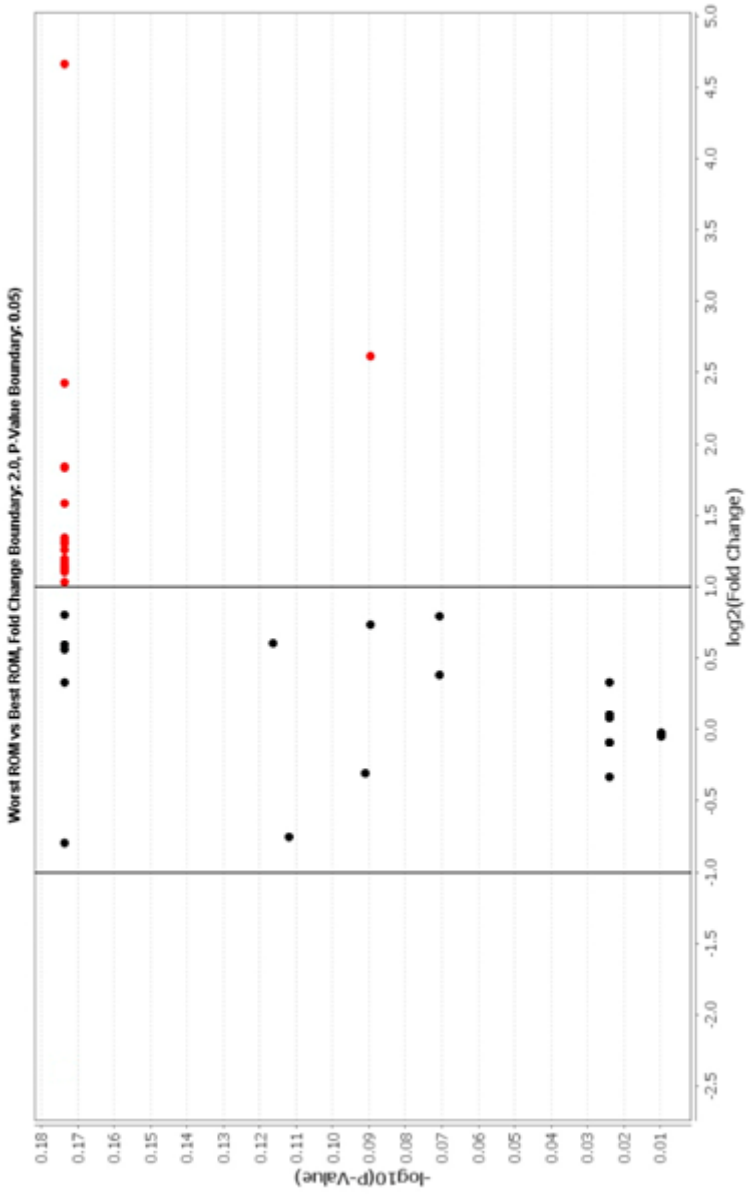
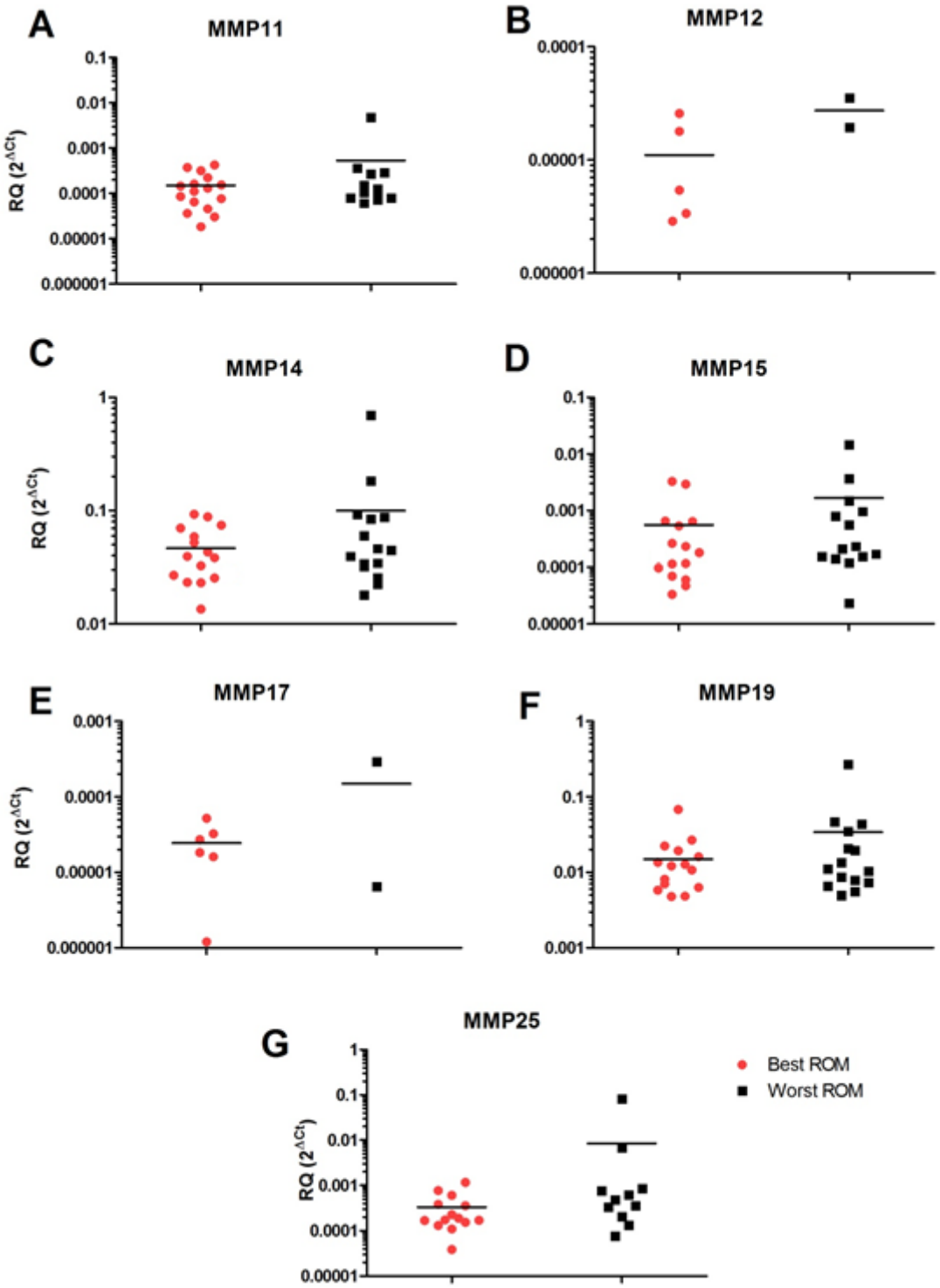


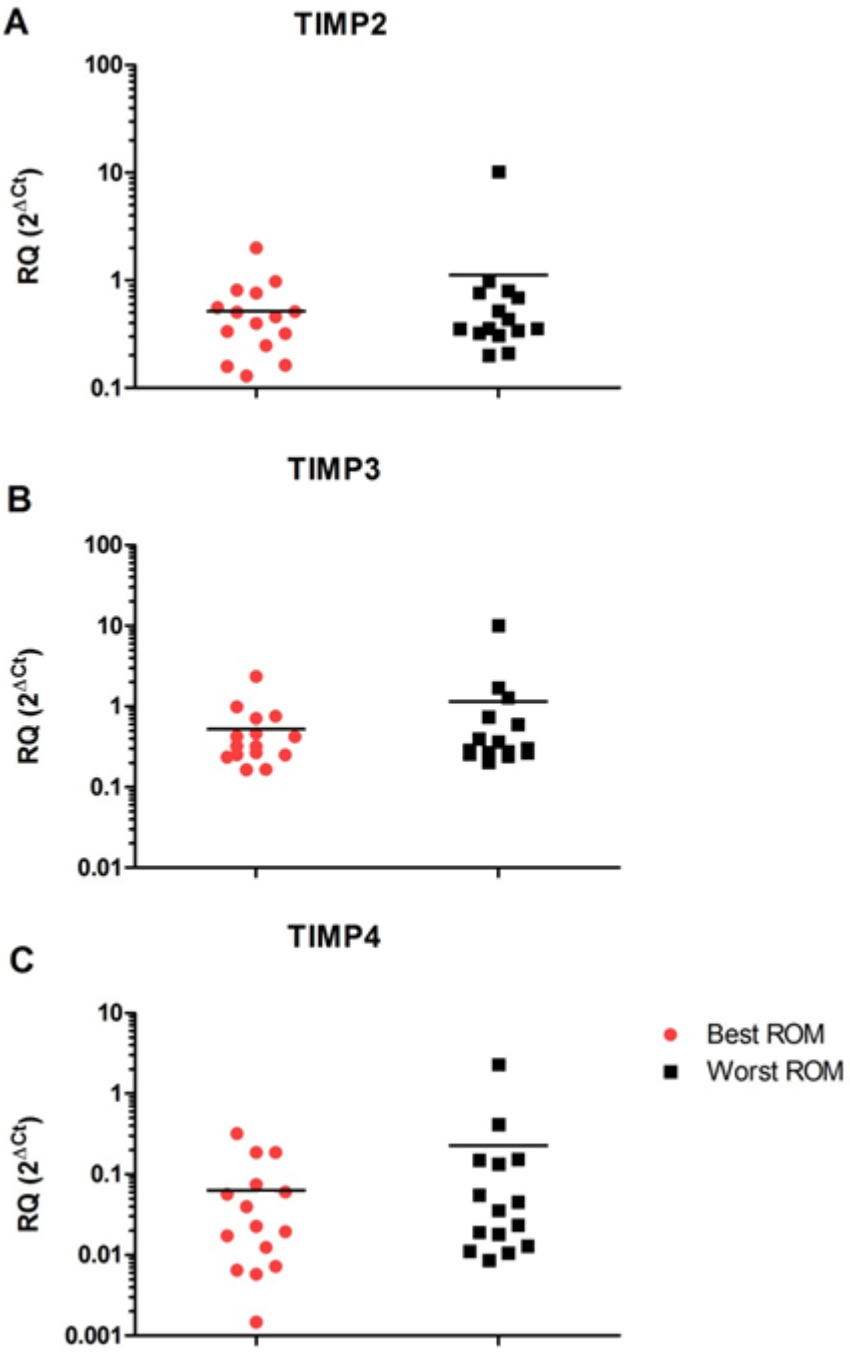
Figure 2: Matrix metalloproteinases (MMP) gene expression in synovial samples divided into 'worst' and 'best' range of motion.



MMP – matrix metalloproteinases; RQ (2^-ΔCt) – relative quantification of 2^- Delta Ct

*mean values represented by the black line

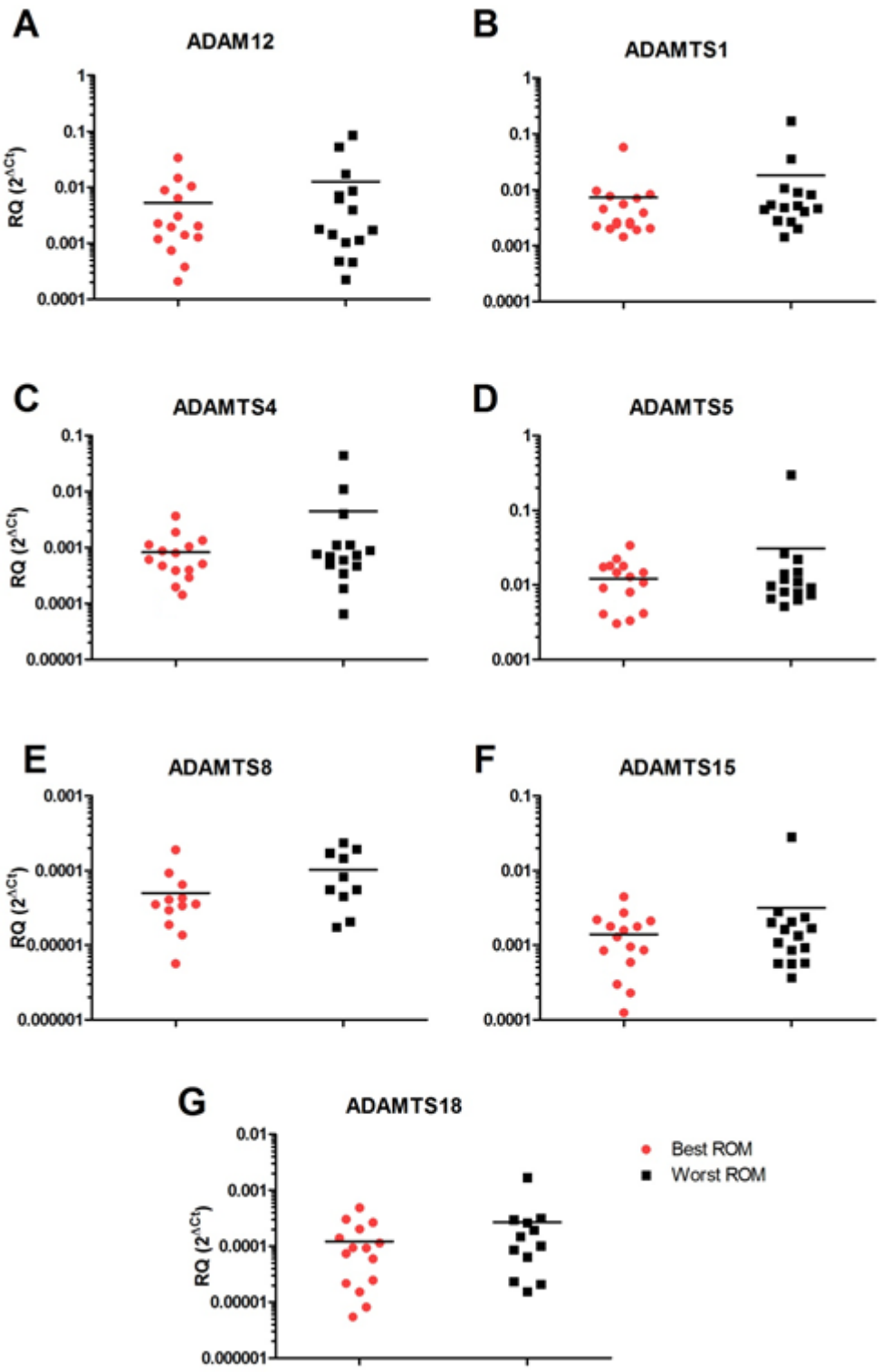
Figure 3: TIMP gene expression in synovial samples divided into 'worst' and 'best' range of motion.



RQ (2^-ΔCt) – relative quantification of 2^- Delta Ct; TIMPS - Tissue Inhibitors of Matrix Metallo Proteinases

*mean values represented by the black line

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



ADAM - A Disintegrin and Metalloprotease; ADAMTS - A Disintegrin and Metalloprotease with Thrombospondin Motif ; RQ (2^-ΔCt) – relative quantification of 2^- Delta Ct;

*mean values represented by the black line

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 PCS 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

Supplementary Table 1: Full list of genes tested

ADAMTS	MMPS	TIMPS
ADAM12-Hs01106101_m1	MMP1-Hs00899658_m1	TIMP1-Hs00171558_m1
ADAMTS1-Hs00199608_m1	MMP10-Hs00233987_m1	TIMP2-Hs00234278_m1
ADAMTS10-Hs00372835_m1	MMP11-Hs00171829_m1	TIMP3-Hs00165949_m1
ADAMTS12-Hs00229594_m1	MMP12-Hs00899662_m1	TIMP4-Hs00162784_m1
ADAMTS13-Hs00260148_m1	MMP13-Hs00233992_m1	
ADAMTS14-Hs00365506_m1	MMP14-Hs00237119_m1	
ADAMTS15-Hs00373520_m1	MMP15-Hs00233997_m1	
ADAMTS16-Hs00373526_m1	MMP16-Hs01095537_m1	
ADAMTS17-Hs00330236_m1	MMP17-Hs00211754_m1	
ADAMTS18-Hs00373501_m1	MMP19-Hs00275699_m1	
ADAMTS19-Hs00999225_m1	MMP2-Hs00234422_m1	
ADAMTS2-Hs00247973_m1	MMP21-Hs00377680_m1	
ADAMTS3-Hs00610744_m1	MMP23A;MMP23B-Hs00270380_m1	
ADAMTS4-Hs00192708_m1	MMP24-Hs00198580_m1	
ADAMTS5-Hs00199841_m1	MMP25-Hs01554789_m1	
ADAMTS6-Hs01058097_m1	MMP26-Hs00983740_m1	
ADAMTS7-Hs00276223_m1	MMP27-Hs00223193_m1	
ADAMTS8-Hs00199836_m1	MMP28-Hs01020031_m1	
ADAMTS9-Hs00172025_m1	MMP3-Hs00968308_m1	
	MMP7-Hs01042795_m1	
	MMP8-Hs01029057_m1	
	MMP9-Hs00957562_m1	