Abstract

Background. We hypothesised thrombospondin-4 (TSP-4), a molecule mediating pain sensitisation in peripheral nerve injury, is associated with pain sensitisation in OA.

Methods. A cross-sectional study of clinical, imaging and fluid biomarkers from knee OA participants was conducted. TSP-4 was assessed by immunohistochemistry (IHC) for OA tissue samples and by ELISA in serum samples. Type II collagen degradation products (CTX-II), linked to OA structural damage, was determined from urine samples. A general linear model (GLM) was used to: a) investigate how patient-reported WOMAC (Western Ontario and McMaster Universities OsteoArthritis Index) pain/stiffness subscales and pain sensitisation measured by painDETECT, related to the Hospital Anxiety and Depression Scale (HADS), structural damage quantified from MRI and X-rays, CTX-II and TSP-4; b) how TSP-4 related to structural damage. We used linear discriminant analysis (LDA) to determine a classifier for pain-sensitisation from clinical and wet-biomarkers.

Results. TSP-4 was expressed in cartilage, bone marrow lesion (BML) and synovial tissue from OA samples. Upregulated TSP-4 protein was observed in cartilage, synovial tissue and BMLs in a perivascular distribution and in fibrotic tissue. Serum TSP-4 was significantly higher ($p=0.001$) in those with pain sensitisation (painDETECT level ≥ 19) compared with non-sensitised participants. Serum TSP-4 was significantly increased with Hoffa's synovitis ($p<0.001$) and number of BMLs ($p<0.001$ to $p<0.05$). LDA provided classification accuracy of 80% for pain sensitisation using TSP-4, CTX-II and HADS, supporting the biopsychosocial model of pain in OA.

Conclusion. Our data suggests TSP-4 is associated with pain sensitisation in OA and is a biomarker stratifying for pain sensitisation. (250 words).

Keywords: Osteoarthritis, Pain, Sensitisation, Fluid biomarker, Magnetic Resonance Imaging, X-ray, Thrombospondin-4, CTX-II

Thrombospondin-4 correlates with MRI measures of structural damage and pain sensitisation: a new biomarker in knee osteoarthritis

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Conclusion. Our data suggests TSP-4 is associated with pain sensitisation in OA and is a biomarker stratifying for pain sensitisation. (250 words).

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Introduction

Osteoarthritis (OA) is a condition affecting the whole joint, causing disability and impairment in millions of people worldwide [1]. Pain is one of the main symptoms of OA, but many people suffering from the condition experience symptoms despite treatment with analgesic drugs and/or joint replacement surgery [2]. There is a huge unmet need to better understand the mechanisms of pain in OA pathophysiology, which may help translate to improved treatments in the future.

Recent work from several groups has suggested that pain sensitisation is a prominent feature of pain in OA and is recognised in the knee [3], hand [4, 5] and hip [6]. Although people with OA are known to have heightened pain sensitivity, including assessment with clinically relevant methodologies such as quantitative sensory testing (QST) [7] and questionnaires including the painDETECT system [8], the biochemical drivers of pain sensitisation in OA pathophysiology are not well understood. Assessment of central sensitisation and neuropathic pain in hand/knee OA have also demonstrated sensitisation and neuropathic elements in OA-related pain [9, 10].

One candidate mediator of pain sensitisation in OA is thrombospondin-4 (TSP-4) [11]. Previous studies have shown that nerve damage such as peripheral nerve injury induces increased expression of TSP-4 in the spinal cord and dorsal root ganglia [12]. Recently, TSP-4 has been shown to act on sensory afferent terminals via voltage-gated calcium channels in the dorsal spinal cord to promote excitatory synaptogenesis and central sensitisation [13,14], suggesting that TSP-4 is a strong candidate for neuropathic pain states observed in OA. Previous data from human OA studies showed that TSP-4 is upregulated in human OA cartilage [15] and bone marrow lesions (BMLs) [16]. TSP-4 elevation may be upregulated in OA as part of repair processes but also lead to nerve growth and the development of pain sensitisation.

We have previously shown that type II collagen degradation product (CTX-II) levels are associated with structural damage [17] with a moderate relationship to reported pain (WOMAC). Based on our previous studies in BMLs showing upregulation of tissue breakdown and pain sensitisation genes [16], we hypothesised that increased levels of TSP-4 are associated with higher reported pain and central sensitisation in OA. In this new analysis, our primary aim was to investigate whether variability in pain scores (WOMAC pain and painDETECT) could be described by clinical measures (age, sex, BMI and HADS) with the addition of the fluid biomarker TSP-4, and if there is a classifier that distinguishes people who are sensitised for pain as determined by the painDETECT score. If this was the case, then readily obtainable clinical information combined with fluid biomarkers may provide an objective measure of sensitisation for pain stratification that is appropriate for routine clinical use. Since we previously found TSP-4 to be upregulated in BMLs [16] we also performed an exploratory analysis to assess if serum TSP-4 was related to structural damage. Secondary aims with the sub-groups of patients for which knee images were available (MRI or plain x-ray), was used to investigate how strongly TSP-4 levels were related to pathological features of structural damage, and whether these parameters provided significant additional information for describing variability in pain scores. Analysis with WOMAC stiffness was also included for completeness as this is a major clinical feature in addition to pain, and we aimed to assess how completely, or not, pain and stiffness scores could be represented without recourse to acquiring imaging data. Our study demonstrates that TSP-4 is a biomarker linked to knee OA pain sensitisation. TSP-4 could be used as a wet biomarker to improve pain stratification in OA for patients requiring medical or surgical management and develop novel therapeutics for this condition.

Methods

Participants

Participants were recruited either as early OA undergoing usual care with analgesics (e.g. NSAIDs, opioids, and/or physical therapies), or as advanced OA scheduled for total knee joint replacement surgery (TKR) surgery of their most affected knee in our cross-sectional study from 2013-2024. The sample size was mainly determined by the original CTX-II study for which there was a target detecting wet biomarker CTX-II, structural and pain differences between groups of early and advanced OA patients [17]. Previous work in our group showed that to detect significant differences in pain between advanced and early OA groups, a recruitment target of N=78 in the advanced OA group and N=42 in the early OA group was required ($p<0.001$). The sample size was then interrogated for wet biomarkers including CTX-II and serum saved for future studies. The current study includes N=85 participants with advanced OA who required joint surgery, N=43 early OA (defined by early Kellgren-Lawrence radiographic scoring of 0-2), requiring medical management only, and N=10 healthy controls. Although we present a comparison of characteristics of early versus advanced OA patients in this current study, the main aim was to investigate prediction of pain and stiffness scores across the whole cohort in relation to our additional new analysis of the blood biomarker TSP-4 (N=120), hence a convenience sample size. The n=10 controls were included for an indicative measure of TSP-4 in healthy volunteers but not used in the statistical analysis due to the limited sample size and age range available at this point. Ethical approval was granted by the London Central REC (12/LO/1970).

MRI and MOAKS

Imaging data was acquired from the most affected knees of both early and advanced OA patients. Knee MRI acquisition, scoring with MOAKS [18] and tissue analysis were performed as previously described [16, 17]. Semi-automated analysis of clinically acquired knee radiographs was performed to obtain joint-space measures, with the Kellgren Lawrence grade based on the original definitions [19]. Further details are provided in supplementary figures 1 and 2 for the MRI and X-ray image analyses as well as for the bone tissue analysis of TSP-4 levels.

T1w and Intermediate Weighted (IW) MRI (17) was performed on the most affected knee using a 3T MRI system with a dedicated 8 channel knee coil. Images (see supplementary Figure 1) were assessed for structural damage with consensus scores of two radiologists as reported in (17) using the MRI Osteoarthritis score (MOAKS) (18), for cartilage damage (CD), bone marrow lesions (BML), osteophytes (Ost), Hoffa's synovitis (Hoff_Syn) and effusion synovitis (Eff_Syn). The total number of lesional regions (nCD, nBML, nOst) were summed from all anatomical sub-regions of the MOAKS analysis for the main analysis of assessing variability in WOMAC pain and stiffness. The MOAKS scores from individual anatomical regions were used for a more detailed sub-analysis investigating variability in serum TSP-4 levels as a function of structural damage.

Joint space (JS) measures

Clinically acquired patient knee x-rays were analysed using ImageBiopsy Lab (IBL, Vienna, Austria) JSx software v1.16, which provided for semi-automatically determined joint-space (JS) measures (see supplementary Figure 2): JS average (JSA), JS width (JSW) and minimum JS height (minH) in medial and lateral joint compartments and an overall Kellgren-Lawrence score (KLG). Since these JS scores

are highly correlated, principal component analysis (PCA) was applied to all six measures and produced two principal components, PC_JS_medial and PC_JS_lateral, which had a correlation |R| > 0.9 to their respective individual measures. These PCs were used in all statistical analyses. The KLG score was based on the original definitions (19) and was reduced to a KLG_low for grades 0,1,2 and KLG_high for grades 3 and 4. Thus KLG_high represents definite joint narrowing, and at minimum some sclerosis and possible deformity.

Tissue

Tissue was harvested as described in (16), with full informed consent from participants with knee OA as defined by American College of Rheumatology (ACR) criteria and who were undergoing TKR. A total of 10 participant samples were analysed containing cartilage and bone, and that included BMLs. Participants had undergone MRI of the target knee within 3-6 weeks prior to TKR and regions within the tibia demonstrating BMLs were confirmed independently by two Consultant Radiologists using the MRI to aid BML localisation within the subchondral bone (17). A further 10 synovial samples were analysed from OA tissue obtained from a study REC number 179325, with Ethical Approval granted by the London-Harrow Research Ethics Committee.

Immunohistochemistry for thrombospondin-4 (TSP-4)

Sections of cartilage, bone and synovium were sectioned with a thickness of 5µm (Leica RM2255, Milton Keynes, UK). Primary Polyclonal Goat TSP-4 antibody (R&D systems, AF2390) in 1% BSA was applied at dilution 1:500 to the cartilage and bone sections and at 1:2000 dilution to the synovium sections. Prior to primary antibody incubation, the endogenous peroxidase activity was blocked with 1% hydrogen peroxide. Slides were then incubated overnight at room temperature. A secondary

HRP-conjugated anti-goat antibody (Abcam, AB6885) was applied at dilution (1:400) for 2 hours at room temperature. All slides were developed with 3,3′-diaminobenzidine (DAB) solution (Abcam ab64238) at room temperature for 2 minutes and counterstained with Haematoxylin.

Fluid biomarkers - Serum Thrombospondin-4 (TSP-4) and CTX-II

TSP-4 ELISA (ELH-TSP4) kits were purchased from RayBiotech (Tebubio, UK). Serum samples were diluted 100-fold, and the assay was incubated with biotinylated anti-human TSP-4 detection antibody, HRP-Streptavidin, and TMB substrate. Absorbance was read at 450nm with the Synergy LX (BioTek) plate reader. Analyses were conducted according to manufacturers' instructions for quantification against a reference calibration curve. Type II collagen degradation products, CTX-II were assessed as described previously [17].

Statistical methods

This study included clinical, imaging (MRI and plain radiography) and wet biomarker data in participants with early and advanced OA. Since all data types were not available for all subjects, the maximum number of patients with all data-types was used within each subgroup analysis.

Pearson's correlation was performed between continuous variables, and the Independent-Samples Jonckheere-Terpstra (ISJT) Test used to assess trends across ordinal variables as a precursor to General Linear Model analysis. Tests for significant differences between subgroups was assessed with Mann-Whitney U-test for continuous and ordinal variables, and Kruskal-Wallis for testing across multiple groups.

To assess how WOMAC pain/stiffness and painDETECT scores relate to clinical and structural damage variables we used a GLM (the “mixed-GLM” of SPSS) applied to the maximum amount of patient data for which all parameters were available: N=118 for clinical scores, N=90 when including MRI parameters and N=93 when including x-ray image data. The GLM was used to generate a prediction of the target variable with the linear form containing ordinal and continuous variables. The clinical group was not included as a factor in the GLM since we are interested in the full continuum of disease processes. An initial GLM analysis was performed on the clinical and fluid biomarker data (age, BMI, HADS, sex, TSP-4 and CTX-II) for which there were n=118 participants with all these available measures. The most significant parameters for describing variability in the pain, namely painDETECT and WOMAC pain and stiffness scores (used directly as target variables) were then taken forward for an analysis with either MRI measures of structural damage (n=90 participant datasets available) or with planar x-ray measures of joint space and Kellgren-Lawrence scores (n=93), to assess whether structural parameters were of additional significance in describing pain variability.

To assess the measured parameters that best described the variability in TSP-4 we used a GLM applied to the MRI data using the raw MOAKS sub-scores. Scores from individual anatomical regions were used in preference to the summed MOAKS parameters since we hypothesised that if TSP-4 originates from sites of structural damage there may be variable contribution from each region. The GLM was applied with synovitis scores as the base variables to which all the MOAKS sub-scores for the different structural characteristics (e.g. Ost, BML, CD) were added in turn.

To investigate whether we could develop a fluid biomarker that classified patients with a clinical measure of pain sensitisation (i.e. painDETECT ≥19), we applied a stepwise

linear discriminant analysis (LDA) to the clinical scores (BMI, Age, sex, painDETECT, HADS) and fluid biomarkers (TSP-4, CTX-II). A leave-one-out assessment was made to provide a more representative measure of the classification accuracy, and the accuracies of combined markers compared to the individual ones.

All statistical assessments were made using IBM SPSS Statistics software v. 29.

Results

Tissue staining for TSP-4

TSP-4 was expressed in the mid-and deep-zones of articular cartilage within chondrocytes and extracellular matrix (ECM), and in a perivascular distribution in synovial tissue (Figure 1). Within the subchondral bone OA-BMLs, TSP-4 staining intensity was increased in a perivascular pattern and within fibrotic areas of BMLs (Figure 1). Staining within synovial tissue and bone showed TSP-4 expressed in the vascular matrix of blood vessels. There was absence of staining with negative controls using isotype-specific immunoglobulin controls (data not shown).

Clinical groups and pain sensitisation

Table 1 shows the mean and range for all parameters used in the analysis across all participants in the study and indicates which parameters are significantly different (Mann-Whitney U-test) between clinical (early versus advanced OA) and painDETECT (non-sensitised compared to those sensitised who have painDETECT ≥ 19 respectively) groups, and sex related differences. The clinical OA groups were significantly different (at Bonferroni corrected $p < 0.003$) for WOMAC pain/stiffness scores and most of the structural damage scores (nBML, nCD, nOst, KLG), but depression (HADS) and the painDETECT sensitisation measures were not

significantly different. TSP-4 and CTX-II were also significantly different between clinical (early and advanced) OA groups. Conversely, participants who showed central sensitisation according to painDETECT ≥ 19 had significantly higher WOMAC scores and TSP-4 levels than those not sensitised, but there were no significant differences in structural damage scores for MRI and X-ray derived parameters or in CTX-II levels. There were also no differences in BMI/age between the two painDETECT groups.

We also investigated whether WOMAC and painDETECT scores related to therapeutic drug use. Of 125 patients for which we had treatment details, N=16 were not taking painkillers; N=75 were taking one or more of analgesics, NSAIDS or anti-depressants; N=33 were taking opioids. There were no significant differences in WOMAC pain or stifness, or in painDETECT, across these three groups. Drug treatments were not considered further in the analyses.

Assessment of TSP-4 variability

TSP-4 is strongly related to the clinical group, being significantly higher in advanced OA participants who exhibit more structural damage (Table 1) but shows no significant variation with age (Table 2). Our histopathological analysis of the TSP-4 location in OA knee (Figure 1) and the correlation results of Table 2, suggests that TSP-4 may directly relate to the damage that is quantified by the MOAKS score. In relation to MRI parameters the most significant variation is with Hoff_Syn (p=0.006), with parallel but less significant variation with Eff_Syn (p=0.012) (Figure 3B). We performed a GLM analysis with Hoffa's synovitis as a base parameter to which we included all the separate sub-scores for MOAKS for each type of structural damage type. With Hoff_syn in the model there was no significance for Eff_syn, or for the nOst and nCD anatomical subscores when added as separate groups. However, the nBML

anatomical region subscores, and specifically those from the patella and tibia, showed a significant contribution to TSP-4 variability ($p<0.04$, $F>4$); the subspinous region score was not significant. Figure 3C shows TSP-4 predicted from a GLM analysis that includes Hoff_syn and the separate MOAKS assessment of number of BMLs in the patella, femur, tibia and trochlear along with their individual p and F-values.

In terms of structural damage, increased TSP-4 levels were most strongly associated with increased Hoffa-synovitis, and secondly with numbers of BMLs, with the patella and tibial region measures providing the most significant contribution to TSP-4 variability (Figure 3). Figure 3 shows our prediction of serum TSP-4 levels directly from the number of OA-BMLs and the synovitis score, thereby suggesting a biological and mechanistic link between structural damage, repair mechanisms and pain sensitisation.

General linear model analysis of WOMAC and painDETECT scores

A GLM of clinical and imaging groups was performed (see Supplementary Tables 1 and 2) to investigate which parameters best predicted the WOMAC (pain and stiffness) and painDETECT scores. For WOMAC pain, only BMI ($p<0.001$, $F=24.7$), TSP-4 ($p=0.019$, $F=5.5$) and HADS ($p<0.001$, $F=16.6$) were significant covariates, and when using these parameters alone the predicted pain score is shown in Figure 2A. For the data subsets with imaging data (MRI or x-ray), TSP-4 showed greater significance and effect size than any individual structural imaging parameter. When individual imaging parameters were examined with TSP-4 also included in the model, only KLG still showed a significant additional contribution ($p=0.004$, $F=8.5$), but the predicted variability was no better described than as shown in Figure 2A. In considering

confounders, there were no significant differences in WOMAC subscales and use of analgesics: NSAIDs, opioids or not using analgesics (using Kruskal Wallis testing).

For predicting painDETECT from clinical parameters and TSP-4, only HADS ($p<0.001$, $F=31.2$) and TSP-4 ($p<0.001$, $F=11.4$) were significant as shown in Figure 2B. In the imaging data subsets, TSP-4 showed greater significance ($p=0.004$, $F=8.8$) than the structural parameters, with only nCD still significant ($p=0.032$, $F=4.8$) when TSP-4 ($p=0.002$, $F=5.5$) was included.

For WOMAC stiffness, log BMI ($p=0.05$, $F=3.7$), TSP-4 ($p<0.001$, 14.4) and sex ($p<0.001$, $F=12.5$) were predictors as shown in Figure 2C. In the imaging data subsets, TSP-4 was more significant ($p=0.007$, $F=7.6$) than any of the imaging parameters added individually. For TSP-4 combined with an imaging parameter only the PC of JS_lateral was significant ($p=0.003$, $F=9.6$) and gave a model with improved description of WOMAC stiffness as shown in Figure 2D.

Of note, the WOMAC scores were strongly correlated to BMI, HADS and a variety of structural damage scores as well as to TSP-4 levels. In contrast, painDETECT correlated to pain, stiffness and depression scores as well as to TSP-4, but did not correlate to measures of structural damage. CTX-II was mostly correlated with structural damage measures as previously shown in (17) but also correlated to TSP-4 levels. TSP-4 levels were correlated to measures of both pain and structural damage.

Prediction of pain sensitisation

A stepwise linear discriminant analysis (LDA) across the n=106 participants that had clinical (Age, HADS, BMI, sex) and both fluid biomarkers reduced to the combination HADS, CTX-II and TSP-4 as effective classifier parameters, with an 80.2% overall

classification accuracy, and 78.3% accuracy in a leave one out validation. This reduces to 70.8% overall accuracy and 68.9% cross validation accuracy for CTX-II combined with TSP-4. In comparison, individual parameters all had lower accuracies: WOMAC_P, 73.4%; WOMAC_S, 66.4%; HADS, 66%; CTX-II, 44.6%; TSP-4, 66.1%. TSP-4 levels are strongly correlated to structural damage (Table 2), including urinary CTX-II, as shown in Figure 4A, which also demonstrates that participants with higher levels of TSP-4 are more likely to be associated with high painDETECT scores that indicate sensitisation. A GLM of this data indicates that TSP-4 is significantly correlated to CTX-II ($p<0.001$) and significantly different ($p<0.001$) between the sensitised and non-sensitised groups, with age, sex and BMI not significant factors. In the graphical presentation correlation coefficients are R=0.41 and 0.32 for PD high=0 and 1 respectively. In Figure 4B we see there is no correlation between HADS and TSP-4, indicating depression is an independent factor in relation to pain sensitisation, with HADS significantly different ($p<0.001$) between the two groups.

Discussion

Our analysis demonstrates that elevated serum levels of TSP-4 are not only an independent predictor of higher WOMAC pain and stiffness scores, but also a potential biomarker that can indicate people with OA who have progressed to a state of central sensitisation with high painDETECT scores. Serum levels of TSP-4 are significantly higher (p = 0.001) in the participants who are sensitised according to painDETECT stratification (i.e. a score ≥19). Since there were no significant differences between the two painDETECT groups for either the MRI or x-ray parameters, our data suggests that TSP-4 is a marker of pain sensitisation that is independent of the key imaging features of structural damage and has the potential advantage of being a quantitative objective marker as compared to the subjective nature of pain questionnaires (20).

Although serum TSP-4 levels correlate with urinary CTX-II, which can be considered a fluid biomarker of structural damage, pain sensitised participants have significantly higher TSP-4 levels for a given level of CTX-II. When these two biomarkers are combined with the HADS score (which is also strongly related to pain perception) we can obtain a high classification accuracy for pain sensitisation of 80% using an LDA. Thus, it may be possible to obtain an assessment of pain sensitisation that is directly related to its biomechanical and biological origins. Such a measure may also have the advantage of being more objective than self-reports of pain, and easier to incorporate into therapeutic drug trials than extensive questionnaires and imaging measurements.

TSP-4 expression is absent in normal cartilage, but significantly raised in early OA, increasing with OA severity [15] and may have a functional role in cartilage extracellular matrix repair processes [21]. Elevated TSP-4 is found in OA-BMLs, but not elevated in normal bone [16]. Normal bone development occurs via ossification of cartilage and TSP-4 has been localised to transient cartilage in bone and is possibly associated with neovascularisation during ossification [21].

There is an association of TSP-4 with a variety of pathologies and tissue remodelling processes in different anatomical sites, and with relevance to pain, it has been discovered to have a role in synaptogenesis [22]. Animal models of joint and peripheral nerve damage show TSP-4 elevated in response to damage, or by intrathecal injection, leading to synaptogenesis, spinal hyperexcitability and the development of neuropathic pain [23]. Genetic inhibition of TSP-4 production, or chemical blocking of the TSP-4 pathway e.g. with gabapentin, can inhibit [24] or reverse the development of injury induced allodynia. Nerve growth factor (NGF) expression is associated with angiogenesis in the sub-chondral bone in rheumatoid and osteoarthritis [25] and our own studies indicate that along with TSP-4 elevation, there is also elevation of genes

associated with neurogenesis in OA bone marrow lesions [16]. Elevated TSP-4 may be a direct factor influencing nerve sensitisation in OA, with the potential for therapeutic targeting using drugs such as Gabapentin. Whether this would be early treatment with low-dose gabapentin to prevent the development of chronic neuropathic pain as suggested by Yu *et al.* [24] or would provide a treatment reversing this condition as observed in studies by Park *et al.* [12], is an area of potential investigation. But a recent study has shown a correlation of blood TSP-4 with reported pain levels of patients with herniated discs, with a decrease in TSP-4 protein levels associated with successful operation to relieve pain, and an increase in patients with uncontrolled pain [11]. Hence TSP-4 may be a dynamic marker in relation to pain outcome.

Our findings are consistent with observations that synovitis mediates the association between OA-BMLs and knee pain [26]. Studies have shown that TSP-4 protein expression is increased in cardiovascular, musculoskeletal and neuronal tissue [27]. We have previously identified increased TSP-4 gene expression in BML tissue from OA participants [16]. Cardiovascular disease (CVD) is associated with elevated TSP-4 [27] and the link between OA and CVD is increasingly recognised [28]. Together with the CVD association, persistence of inflammation with recruitment of macrophages to regions of inflammation could be attributed to TSP-4 binding to macrophages [29].

High TSP-4 protein levels may contribute to peripheral sensitisation due to its expression at neuromuscular junctions [30]. TSP-4, which is synthesized in neurons and glial cells following peripheral nerve injury, can also be secreted into the extracellular space on dorsal root ganglion neurons in an autocrine manner with an observed rise in TSP-4 in the DRG [13]. TSP-4 knock down studies in mice also show that TSP-4 is necessary for the development of pain [11]. TSP-4 increases synaptic transmission of excitatory neurones, but decrease the excitability of inhibitory

neurones, thus suggesting the mechanism for progression to pain sensitisation [11]. Injury or inflammation of the peripheral nerve causes upregulation of Cav α2 δ1 in the DRG [31, 32] and spinal cord (33). The gabapentinoids: gabapentin and pregabalin, are Cav α2 δ ligands [33], and Cav α2 δ1 is also the main receptor mediating effects of TSP-4 in the nervous system [31]. Gabapentin has been shown to block the effects of TSP-4 after nerve injury [34] and in a study of patients with hand OA, pregabalin has been shown effective in reducing pain [35], hence in OA may be affecting both peripheral and central pain processing [36]. Genetic deletion of the Cav α2 δ1 also blocks the TSP-4 mediated effects on cytoplasmic Ca2+ [37, 38]. Although effective in treating some patients with symptoms of neuropathic pain, gabapentinoids are ineffective in treating many other patients with pain sensitisation [38]. Since TSP-4 is known to bind macrophages [39], then production of TSP-4 from BML regions could enable its retention within the joint by binding to synovial macrophages [39] and ongoing release into the systemic circulation. Furthermore, treatments which are more specifically targeted to TSP-4 could also be developed for pain sensitisation.

Study limitations are that this is a cross-sectional analysis and a longitudinal study is needed to determine if there is a definable TSP-4 threshold at which people with OA progress to chronic pain. We observed sex differences in pain-sensitisation by painDETECT and sex differences are also seen in healthy individuals [40]. Whilst chronic pain is more common in women, it is unclear if differences in pain sensitivity or response to analgesics relates to genetic, hormonal or psychosocial factors [41].

Conclusion

Elevated serum TSP-4 is associated with pain sensitisation in knee OA. A strength of our work is acquisition of detailed measures of structural damage made from both MRI

and X-ray, and of pain measures and sensitisation. Our data has enabled a detailed analysis to assess the most important factors relating to pain variability in our knee OA group. HADS and TSP-4 (both measures that relate to pain), in combination with CTX-II (a measure of structural damage), showed high classification accuracy of 80% for predicting patients with high painDETECT scores that are most likely to exhibit central sensitisation. Future work will need to assess the utility of these as a biomarker for OA pain sensitisation in longitudinal studies, and for determining whether modulation of TSP-4 levels is achievable as a method to reduce or reverse pain sensitisation.

Author contributions

NS wrote the initial study protocol and associated documents, co-ordinated the implementation of the study, assisted with data analysis. FAH drafted the initial manuscript and co-ordinated the data analysis. FAH, SK, AB and AH contributed to the final study design, data acquisition/analysis, writing and review of the manuscript. AL, VE and RL contributed to the data analysis, writing and review of the manuscript. SK, STK, AN and AB prepared and analysed tissue samples for histology, immunohistochemistry, serological analysis and conducted data analysis. MS contributed to the interpretation of histological samples for the study. All authors contributed to review of the final manuscript.

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The funders did not have any influence on the running of the study, data collection or reporting of results. The views expressed are those of the author(s) and not necessarily those of the funders.

Acknowledgements

We express our gratitude to all patients who participated in this study.

Conflict of interest

None declared

Data availability statement

The data associated with this article will be shared upon reasonable request to the corresponding author.

Table 1 Summary of measured parameters and their group differences

Parameter	N	Min	Max	Average (StDev)	Clinical group (early/advanced)	painDETECT group	Sex
Clinical							
BMI (kg/m^2)	127	18.4	52.1	31.5 (5.7)	0.003*	0.087	0.028
Age (yr)	128	47	88	67 (8.7)	0.006	0.669	0.775
WOMAC_P	128	0	98	53.3 (24.3)	<0.001*	<0.001*	0.101
WOMAC_S	128	0	100	58.0 (28.1)	0.001*	<0.001*	<0.001*
painDETECT	127	0	30	12.6 (6.8)	0.290	-	0.005
HADS	127	0	35	12.9 (7.3)	0.632	<0.001*	0.002*
MRI							
Eff_Syn	93	0	3	1.6 (0.9)	0.023	0.338	0.320
Hoff_Syn	93	0	3	1.1 (0.8)	0.755	0.618	0.381
nBML	93	0	30	10.2 (6.3)	<0.001*	0.996	0.616
nCD	93	1	14	10.1 (3.4)	<0.001*	0.502	0.814
nOst	93	1	18	9.1 (4.0)	<0.001*	0.620	0.453
X-ray							
KLG	100	0	4	2.4 (1.2)	<0.001*	0.945	0.981
PC_JS_Lateral	100	-2.9	2.2	0.0 (1.0)	0.878	0.200	0.667
PC_JS_Medial	100	-2.2	3.5	0.0 (1.0)	0.018	0.594	0.135
Blood biomarkers							
CTX-II (ng/mmol)	112	58.7	1369	383 (246)	<0.001*	0.877	0.017
TSP-4 (ng/ml)	120	0	30	8.3 (6.2)	0.002*	0.003*	0.919

Legend.

Clinical group is comparison of the early versus advanced OA patients. painDETECT group compares those with painDETECT >19 that are most likely sensitised to those not. Sex is comparison of 95 female and 33 male participants. The *p*-values marked * survive a multiplicity correction for 16 comparisons, $p < 0.05/16 = 0.003125$. Note that PC, represents the principle components of the x-ray joint space measures, hence by definition have mean and standard deviation of 0 and 1 respectively.

Table 2 Correlation of pain and stiffness scores and fluid biomarkers, with all measured parameters

		WOMAC Pain	*WOMAC Stiffness*	*BMI*	*Age*	*HADS*	*pain-DETECT*	*Eff_ Syn*	*Hoff_ Syn*	*nBML*	*nCD*	*nOst*	*PC_JS_ lateral*	*PC_JS_ medial*	*KLG*	*CTX-II*	*TSP-4*
WOMAC Pain	*R*	1	0.661**	0.408**	0.019	0.381**	0.491**			0.193	0.376**	0.364**	0.087	-0.141		0.136	0.224*
	p		**<0.001**	**<0.001**	0.830	**<0.001**	**<0.001**	**0.023**	0.591	0.064	**<0.001**	**<0.001**	0.390	0.161	**0.002**	0.152	**0.014**
	N	128	128	127	128	127	127	93	93	93	93	93	100	100	101	112	120
WOMAC Stiffness	*R*	0.661**	1	0.245**	0.006	0.230**	0.387**			0.158	0.316**	0.304**	0.216*	-0.185		0.088	0.266**
	p	**<0.001**		**0.005**	0.945	**0.009**	**<0.001**	0.306	0.354	0.130	**0.002**	**0.003**	0.031	0.066	0.074	0.356	**0.003**
	N	128	128	127	128	127	127	93	93	93	93	93	100	100	101	112	120
pain-Detect	*R*	0.491**	0.387**	0.221*	-0.132	0.403**	1	0.031	-0.070	-0.019	0.178	0.115	0.110	-0.083	0.098	0.008	0.288**
	p	**<0.001**	**<0.001**	**0.013**	0.139	**<0.001**		0.768	0.506	0.853	0.088	0.272	0.274	0.413	0.333	0.935	**0.002**
	N	127	127	126	127	127	127	93	93	93	93	93	100	100	100	112	119
CTX-II	*R*	0.136	0.088	0.205*	0.072	0.074	0.008			0.295**	0.331**	0.313**	-0.036	-0.070		1	0.336**
	p	0.152	0.356	**0.031**	0.452	0.436	0.935	**0.009**	**0.004**	**0.005**	**0.002**	**0.003**	0.733	0.499	0.285		**<0.001**
	N	112	112	111	112	112	112	88	88	88	88	88	95	95	95	112	106
TSP-4	*R*	0.224*	0.266**	0.091	-0.080	0.008	0.288**			0.204	0.225*	0.226*	-0.009	-0.020		0.336**	1
	p	**0.014**	**0.003**	0.327	0.384	0.935	**0.002**	**0.012**	**0.006**	0.054	**0.033**	**0.032**	0.933	0.850	0.465	**<0.001**	
	N	120	120	119	120	119	119	90	90	90	90	90	94	94	95	106	120

Correlation between pain and stiffness scores, and the fluid biomarkers TSP-4 and CTX-II, across the maximum number (*N*) of patients available for each parameter. Continuous variables were analysed with a Pearson correlation and we report the *R* correlation coefficient and significance *p* (significant values shown in bold). A Jonkhere-Terpstra analysis was performed for ordinal variables (Hoff_Syn, Eff_Syn, KLG) to determine if there was a significant increase with value and just a *p* value is reported.

Figure Legends

Figure 1 Localisation of TSP-4 staining within knee joint OA tissue

Subset of knee OA participant samples evaluated for TSP-4 immunohistochemistry of TKR-harvested knee samples, assessing cartilage, bone and perivascular tissue (total analysed: 10 participants mean age 67 yr), and synovial tissue (total analysed: 9 participants and mean age 66 yr).

A). All samples tested were from female participants. Staining within an OA-BML B) and of synovial tissue C), demonstrates presence of TSP-4 within all tissue types. In cartilage, TSP-4 expression is observed in chondrocytes and the extracellular matrix (ECM). In OA-BMLs, TSP-4 is expressed in the matrix of blood vessels and in areas of fibrosis. In synovial tissue, TSP-4 is expressed in the matrix of blood vessels and connective tissue.

Figure 2 Predicted WOMAC pain, painDETECT and WOMAC stiffness, and scores derived from subsets of measured parameters

Predicted pain and stiffness scores determined by a mixed GLM from subsets of clinical, imaging and fluid biomarkers. *p* values provided for those parameters with a significant contribution to the variability for each score.

For N=118 participants using clinical data alone: A) WOMAC pain with logBMI ($p<0.001$,$F=24.7$), HADS ($p<0.001$, $F=16.6$), TSP-4 ($p=0.019$, $F=5.5$), $R^2 = 0.318$; B) painDETECT with HADS ($p<0.001$, $F=31.2$), TSP-4 ($p<0.001$, $F=11.4$), $R^2 = 0.297$; C) WOMAC stiffness with logBMI ($p=0.05$, $F=3.7$), sex ($p<0.001$, $F=12.5$), TSP-4 ($p<0.001$, $F=14.4$), $R^2 = 0.189$.For N=93 patients with clinical plus x-ray image parameters: D) WOMAC stiffness with logBMI ($p=0.001$, $F=10.9$), sex ($p<0.001$, $F=15.9$), TSP-4 ($p<0.001$, $F=17.3$), lateral joint space ($p=0.003$, 9.6), $R^2 = 0.340$.

Figure 3 Variability of TSP-4 with age and synovitis and TSP-4 prediction from MRI parameters.

A) Variation of TSP-4 with age and clinical sub-group. TSP-4 was significantly higher in advanced OA patients compared to mild OA ($p=0.002$). Non-OA controls have similar levels of TSP-4 to mild OA patients, but data are from a significantly younger cohort ($p=0.006$), and so are only included for visual comparison and not used in statistical analyses. B) TSP-4 increases significantly with levels of Hoffa's synovitis ($p=0.006$) and effusion synovitis ($p=0.012$). C) mixed GLM to predict TSP-4 levels from MRI parameters showed significant contributions from Hoffa's synovitis ($p<0.001$, $F=44.4$), and the number of BMLs in the anatomical sub-regions of patella ($p<0.001$, $F=17.7$), tibia ($p=0.002$, $F=9.9$), femur ($p=0.03$, $F=4.9$) and trochlear ($p=0.041$, $F=4.3$), $R^2 = 0.308$.

Figure 4 Prediction of pain sensitisation from fluid biomarkers and depression

TSP-4 correlates with CTX-II, with TSP-4 significantly higher in patients with painDETECT $\geq$ 19 (PDhigh=1 in plots above). B) HADS is significantly higher in patients with painDETECT $\geq$ 19, but does not correlate with TSP-4, B). An LDA of combined TSP-4 and CTX-II had a classification accuracy of 70.8%, with a 68.9% accuracy in a leave one out validation Addition

of HADS to the model increased the classification accuracy to 80.2%, with a 78.3% accuracy in a leave one out validation.

Supplementary figure legends

Figure 1 Representative knee MRI of a 76yr patient with advanced OA

Three image types were acquired at 3T for analysis by MOAKS. A) Sagittal T1w image (TE 15ms, TR 600ms), and B) axial and C) coronal IW images (TE30ms, TR 5000ms and fat suppression). Features assessed by MOAKS are: osteophytes (small black arrows), cartilage degradation (large white arrows), bone marrow lesions (small white open arrows), synovitis (small white arrows).

Figure 2 Automated scoring of knee x-rays for joint space

Image Biopsy Lab software v 1.12 as applied to a knee radiograph of a participant with advanced OA of the knee. Contour splines with landmarks and labels assigned to both medial and lateral compartments of the knee are automatically applied and then manually adjusted for final positioning.

ABBREVIATIONS

ACR: American College of Rheumatology

BMI: Body Mass Index

BML: Bone Marrow Lesion

CD: Cartilage Damage

CTX-II: type II collagen degradation products

Eff-Syn: Effusion Synovitis

ELISA: Enzyme-Linked ImmunoSorbent Assay

GLM: General Linear Model

HADS: Hospital Anxiety and Depression Scale

Hoff-Syn: Hoffa-Synovitis

ISJT: Independent-Samples Jonckheere-Terpstra

JS: Joint Space

JSA: Joint Space Average

JSW: Joint Space Width

KLG: Kellgren Lawrence Grade

LDA: Linear Discriminant Analysis

MOAKS: MRI Knee OsteoArthritis Score

MRI: Magnetic Resonance Imaging

Ost: Osteophytes

PCA: Principal Component Analysis

REC: Research Ethics Committee

TKR: Total Knee Replacement

TSP-4: thrombospondin 4

VAS: Visual Analogue Scale

WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index

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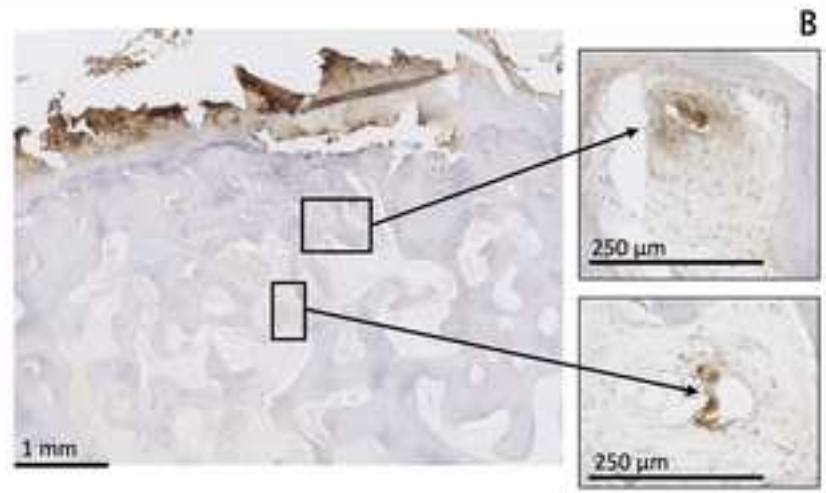
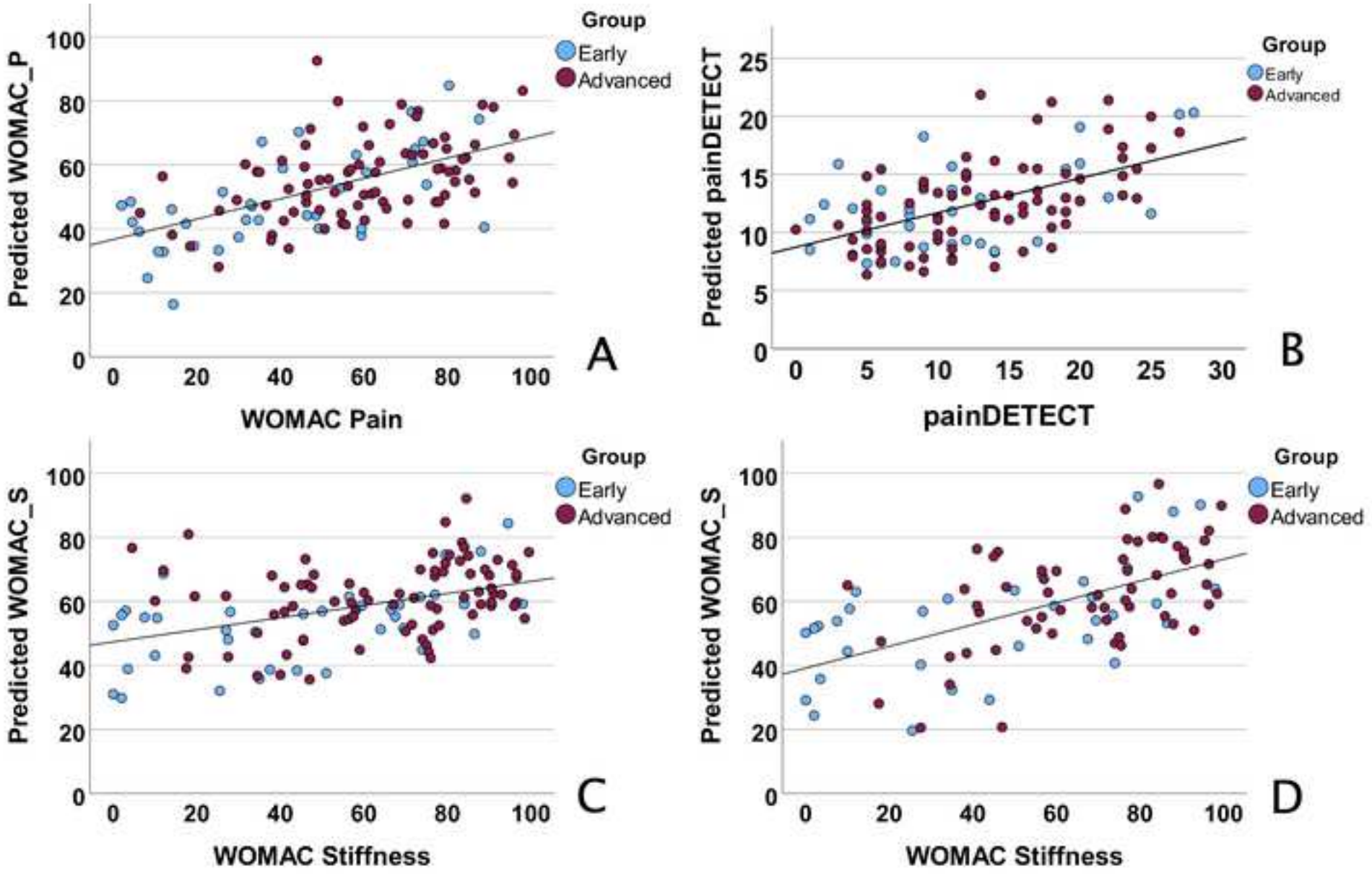
A

BML	CARTILAGE	BONE	PERIVASCULAR	SYNOVIUM	
PA044 1. Age 76	√	√	√	CRY 337 1. Age 76	√
PA069 2. Age 65	√	√	√	CRY 763 2. Age 66	√
PA081 3. Age 67	√	√	√	CRY 370 3. Age 67	√
PA083 4. Age 79	√	X	X	CRY 290 4. Age 79	√
PA116 5. Age 64	√	√	√	CRY 308 5. Age 62	√
PA123 6. Age 74	√	√	√	CRY 726 6. Age 71	√
PA129 7. Age 54	√	√	√	CRY 566 7. Age 52	√
PA125 8. Age 74	√	X	X	CRY 577 8. Age 74	√
PA124 9. Age 55	X	X	X	CRY 764 9. Age 55	√
PA126 10. Age 51	√	√	√		
TOTAL	9/10	7/10	7/10		9/9



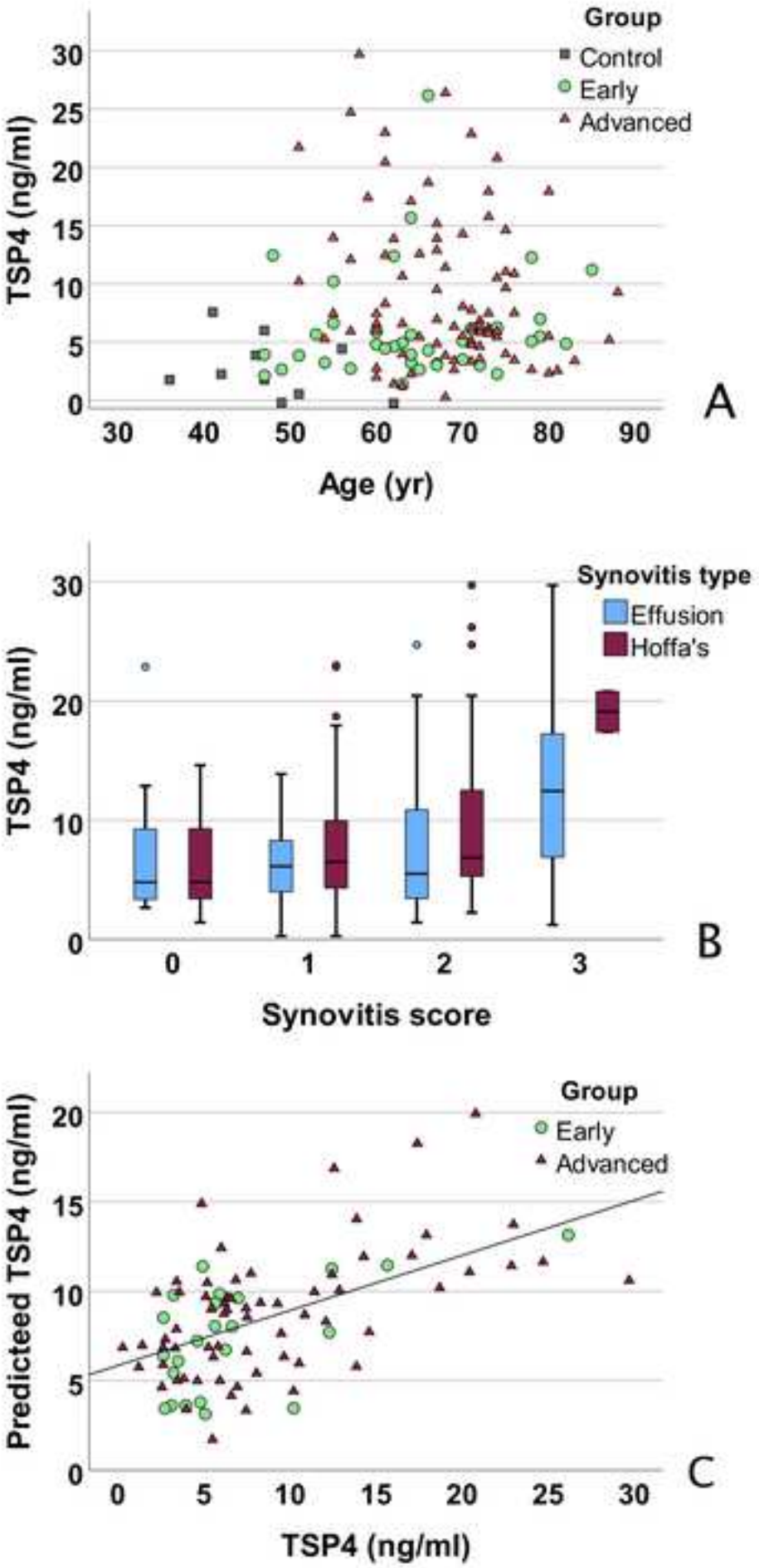
Figure(s)

Click here to access/download;Figure(s);FIGURE 2 Four GLM_pain_painD_stiff.png



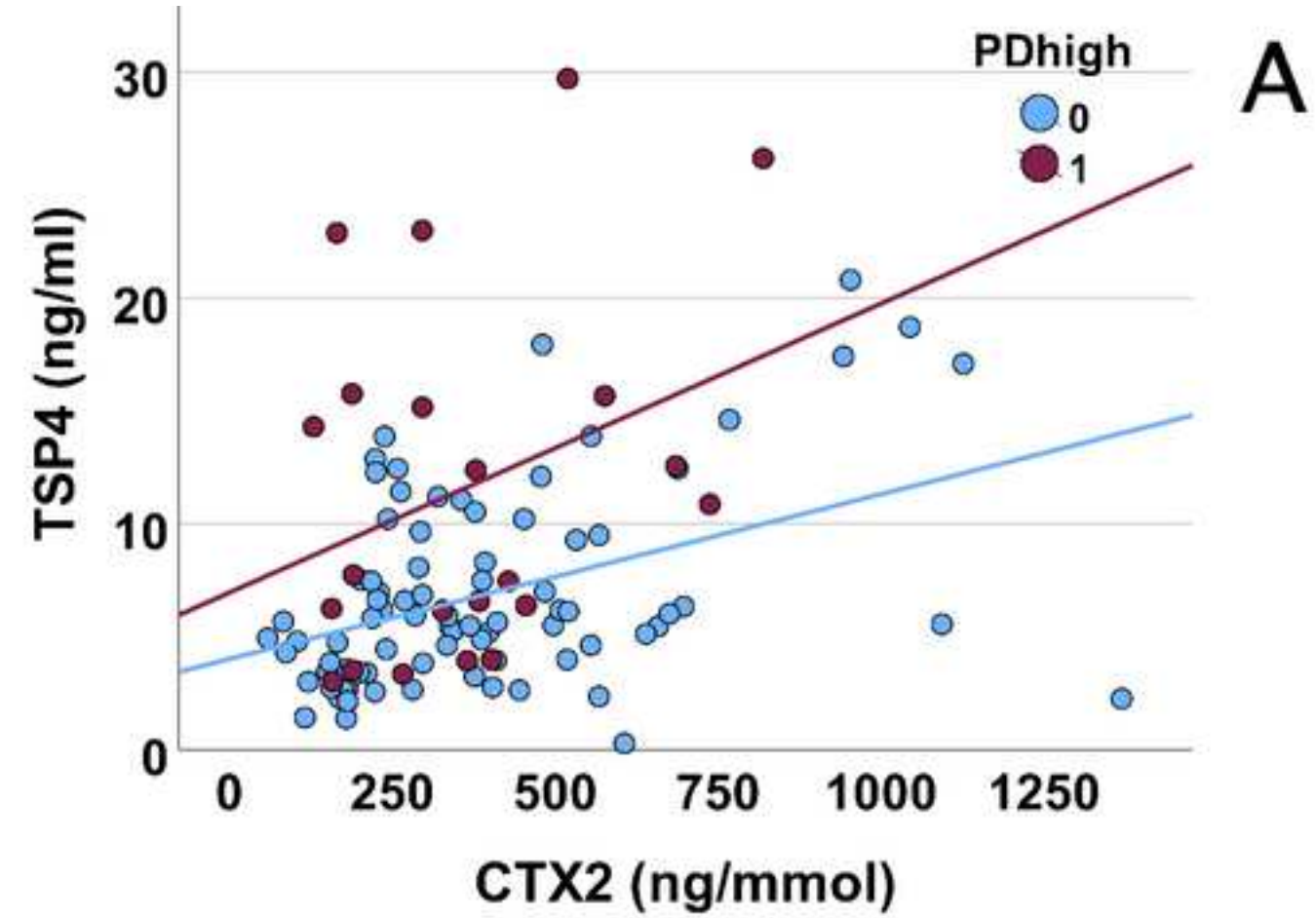
Figure(s)

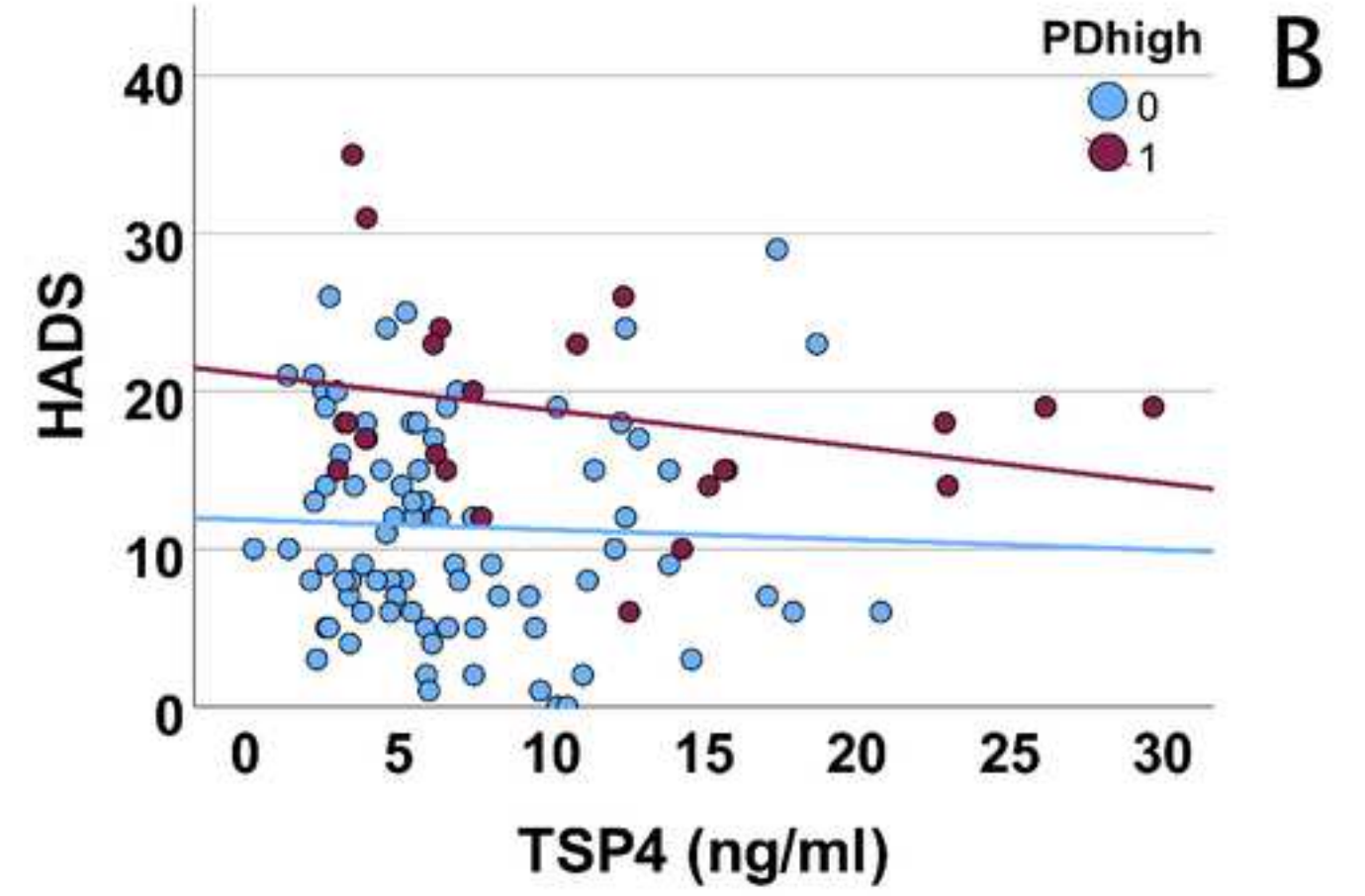
Click here to access/download;Figure(s);FIGURE 3 TSP4 age syn pred.png



Figure(s)

Click here to access/download;Figure(s);FIGURE 4 CTX_HADS_TSP.png





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22 January 2013

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Dear Dr Sofat

Study title:	**Understanding pain perception in osteoarthritis: a mechanistic study in people with knee osteoarthritis (PAPO)**
REC reference:	**12/LO/1970**
Protocol number:	**N/A**
IRAS project ID:	**99426**

Thank you for your letter of 07 January 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Ms Julie Kidd, Juliekidd@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites
NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		26 November 2012
Evidence of insurance or indemnity		01 August 2012
GP/Consultant Information Sheets	2	07 July 2012
Investigator CV		14 October 2012
Participant Consent Form	1.1	07 January 2013
Participant Information Sheet	1.1	07 January 2013
Protocol	1.1	22 November 2012
Questionnaire: Hospitality Anxiety & Depression Scale		
REC application		22 November 2012
Referees or other scientific critique report		30 October 2012
Response to Request for Further Information		07 January 2013

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/LO/1970	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely
PP

Dr John Keen
Chair

Email:Juliekidd@nhs.net

Copy to: *Lisa Clutterbuck, St George's University of London*

Health Research Authority

London - Central Research Ethics Committee

3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

30 December 2022

Dr Nidhi Sofat
Clinical Senior Lecturer/Honorary Consultant in Translational Medicine
St.George's, University of London
1.145 Jenner Wing
Division of Biomedical Sciences,
Cranmer Terrace, London
SW17 ORE

Dear Dr Sofat

Study title:	**Understanding pain perception in osteoarthritis: a mechanistic study in people with knee osteoarthritis (PAPO)**
REC reference:	**12/LO/1970**
Protocol number:	**N/A**
Amendment number:	**Substantial amendment 10**
Amendment date:	**09 December 2022**
IRAS project ID:	**99426**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Completed Amendment Tool [Amendment tool]	1.6	09 December 2022
Letter from funder [Grant award letter]	LEG23010	09 December 2022
Research protocol or project proposal [Protocol]	4	09 December 2022

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Amendments related to COVID-19

We will update your research summary for the above study on the research summaries section of our website. During this public health emergency, it is vital that everyone can promptly identify all relevant research related to COVID-19 that is taking place globally. If you have not already done so, please register your study on a public registry as soon as possible and provide the HRA with the registration detail, which will be posted alongside other information relating to your project.

Statement of compliance

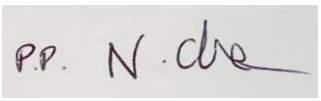
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

HRA Learning

We are pleased to welcome researchers and research staff to our HRA Learning Events and online learning opportunities– see details at: https://www.hra.nhs.uk/planning-and-improving-research/learning/

IRAS Project ID - 99426:	**Please quote this number on all correspondence**

Yours sincerely



Chair

E-mail: londoncentral.rec@hra.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Debbie Rolfe, Joint Research & Enterprise Office St George's, University of London & St George's University Hosp.*

London - Central Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 29 December 2022

Committee Members:

Name	*Profession*	*Present*	*Notes*
Ms Nayema Tahmin	Registered Pharmacy Technician	Yes	
Professor Gareth Tudor-Williams	Consultant in Paediatric Infectious Diseases	Yes	Meeting Chair