Pain mechanistic networks: the development using supervised multivariate data analysis and implications for chronic pain

PAIN

Pain Mechanistic Networks: The Development using Supervised Multivariate Data Analysis and Implications for Chronic Pain

--Manuscript Draft--

Manuscript Number:	PAIN-D-24-00569R2
Full Title:	Pain Mechanistic Networks: The Development using Supervised Multivariate Data Analysis and Implications for Chronic Pain
Article Type:	Research Paper
Section/Category:	Clinical Measurements
Keywords:	pain networks; microRNA; Pain; proteomics; Postoperative Pain; functional outcomes; QST.
Corresponding Author:	Kristian Kjær-Staal Petersen, Ph.D. Aalborg Universitet Aalborg East, DENMARK
Corresponding Author's Institution:	Aalborg Universitet
Order of Authors:	Rocco Giordano
	Lars Arendt-Nielsen
	Maria Carla Gerra
	Andreas Kappel
	Svend Erik Østergaard
	Camila Capriotti
	Cristina Dallabona
	Kristian Kjær-Staal Petersen, Ph.D.

Center for
Neuroplasticity
and Pain

Center for Neuroplasticity and Pain (CNAP)
SMI®, Dept. of Health Science and Technology
The Faculty of Medicine

Aalborg University
Selma Lagerløftvej 249, 9260 Gistrup,
Denmark

Web cnap.hst.aau.dk

Date 20 August 2024

Dear Professor Karen D. Davis

PAIN-D-24-00569
Title: Pain Mechanistic Networks - Development using Supervised Multivariate Data Analysis and Implications for Chronic Pain

Authors: Rocco Giordano; Lars Arendt-Nielsen; Maria Carla Gerra; Andreas Kappel; Svend Erik Østgaard; Camilla Capriotti; Cristina Dallabona; Kristian Kjær-Staal Petersen.

Enclosed, please find the re-resubmission of our study. As you will see in the response letter, all reviewers are satisfied with our revisions, and only a single comment remains to be addressed. We have thoroughly addressed this by providing more detailed illustrations, which has resulted in the addition of several supplementary figures to ensure the sufficient quality required by the reviewer.

On behalf of the authors

Kristian Kjær-Staal Petersen
Associate Professor, M.Sc., Ph.D., D.M.Sc.
Aalborg University, Denmark

(Following please find marked in red changes made in the manuscript)

Reviewer #1

Comment 1: The authors have answered and reformulated the response to comment 1, effectively explaining the possibilities and challenges in DIABLO analysis. The expanded discussion provides a clearer understanding of the application and limitations of DIABLO in pain research, which is a valuable addition to the manuscript.

Comment 2: The response to comment 2, including the integration and rewording of novel text parts, is satisfactory.

Comment 3: The authors have adequately addressed comment 3, with appropriate integration and rewording of the novel text parts.

Comment 4: The response to comment 4, including the rewording of the keyword, is acceptable.

Comment 5: The authors have successfully integrated and reworded the novel text parts in response to comment 5.

Comment 8: The response to comment 8 and its integration into the discussion is satisfactory.

Comment 9: Although I indeed have a different opinion here I can accept the changes the authors made - as a compromise. My point is: QST results are a vehicle to identify sensory changes in patients but do not represent clinical pain. This might be a matter of discussion later one and I am curious about these discussions in the future.

Comments 10-12: The responses to comments 10, 11, and 12 are all acceptable. The authors have integrated the feedback appropriately.

Conclusion: Giordano et al. have made significant improvements to their manuscript, "Pain Mechanistic Networks - Development using Supervised Multivariate Data Analysis and Implications for Chronic Pain." Most of my comments have been addressed effectively, with only minor revisions needed to enhance figure clarity.

Authors response to reviewer based on comment 1-5 + 8-12 + conclusion: We thank the reviewer for

Comment 7: While the authors clarified that the PDFs in the submission document are placeholders and meet the journal's requirements (e.g., 300 dpi), it is essential to address the clarity of the figures in general (not technical issues). Specifically, in Figure 2, the numbers on the x-axis and the individual miRNAs are very small, and similar issues exist with the circle plots in Figure 1. Revising the layout of these figures to increase clarity and readability is crucial, particularly since PAIN is also a print journal.

Authors response to reviewer based on comment 7: In order to improve the quality of the figures and ensure that all readers of PAIN can access the detailed information presented in the images, Figure 1 has now been divided into Figure 1 and Figure 2 and quality of text have been improved. Additionally, the loading plots, which contain more detailed information about the results, have been moved to the supplementary section to ensure that the number of figures and tables match the expectations of the journal. The quality of the figures moved to the supplementary material has been improved. All references in the text to the figures and figure legends have been updated accordingly.

Changes in manuscript main text in "Results" section:

The observed loading values for these variables varied significantly across the blocks, miRNAs (Figure S2), proteins (Figure S3), clinical (Figure S4), and QST Figure S5) indicating differential contributions to the component structure. When investigating 3 groups miRNAs loading values suggested a moderate to strong negative (Figure S2) association with the first component. Notably, variables within this block showed a predominant linkage to the severe clinical pain intensity group outcome. The protein variables presented a narrower range of loading values indicating a weaker negative association with component 1 (Figure S3). The variables in this block were also primarily associated with the severe pain intensity group outcome, though the strength of this association was less pronounced than in miRNAs' block. Clinical variables demonstrated a unique distribution of loading values, with a strong negative association for PCS in the severe pain group and a strong positive association in the non-to-mild pain group (Figure S4). QST variables showed a strong positive (Figure S5) correlation between CPM and PDT in the severe pain and none-to-mild pain groups, respectively. A weak (Figure S5) association was observed for TS in the moderate pain group.

When evaluating 2 groups MiRNA loading values suggested a moderate to strong negative (Figure S6) association with the first component with a predominant linkage to the moderate-severe pain intensity group outcome. The protein variables presented a narrower range of loading values indicating a weaker negative association with component 1, except for GCSF which showed a strong association with the investigated component (Figure S7). Clinical variables demonstrated a unique distribution of loading values, with a strong negative association for PCS in the moderate-severe pain and a weak to medium positive association in the none-to-mild pain group (Figure S8). QST variables showed a medium positive (Figure S9) contribution for CPM in the moderate-severe pain group, a strong contribution of PDT in none-to-mild pain groups, and a medium contribution was observed for TS in the none-to-mild pain group.

Changes in figures and figures legend:

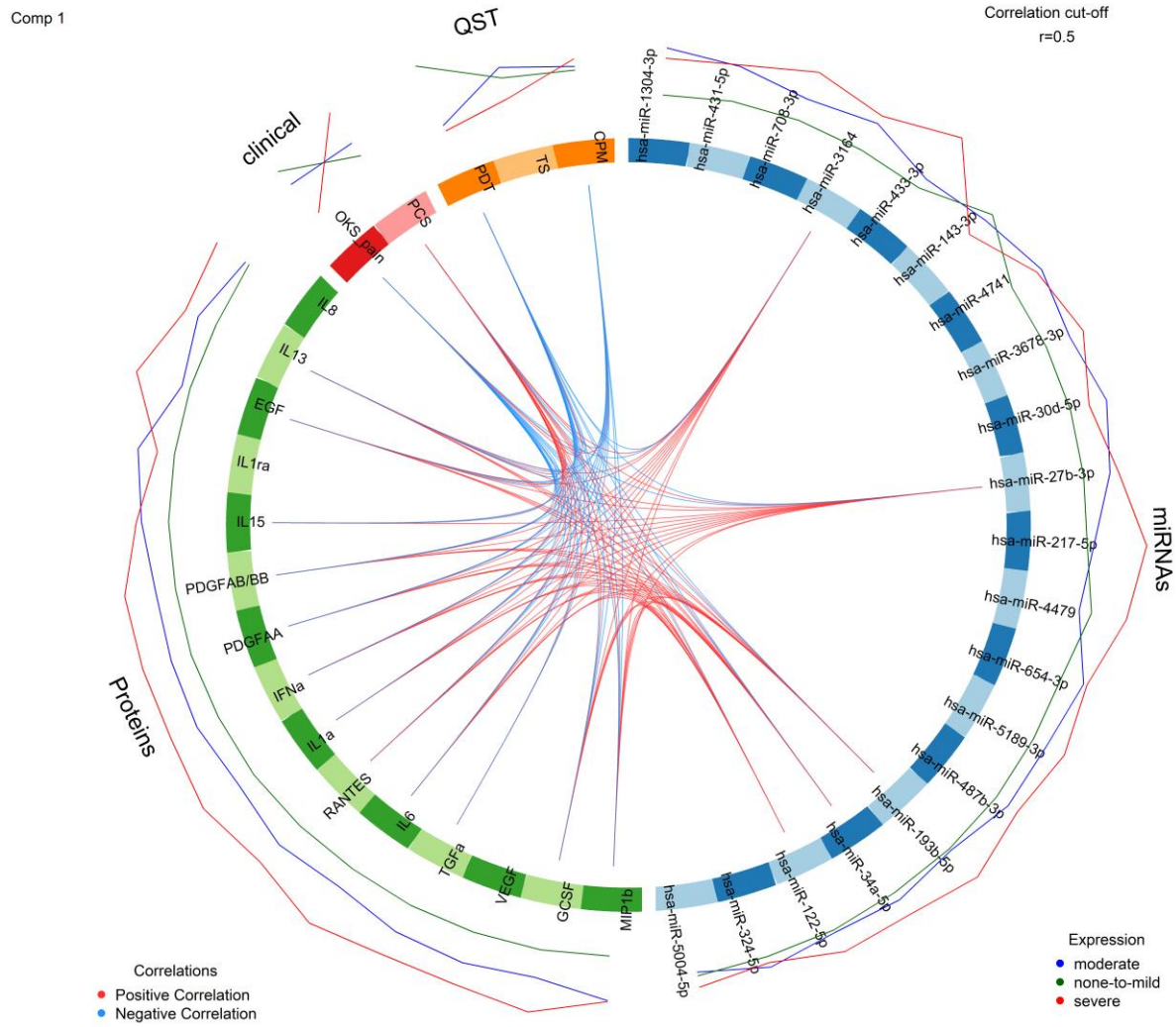


FIGURE 1. Circos plot from multiblock sPLS-DA for component 1 in 3 groups separation. The plots represent the correlations greater than 0.5 between variables selected for all parameters. The internal connecting lines show the positive (red) and negative (blue) inter-correlations. The outer lines show the expression levels of each variable in each sample group, none-to-mild (dark green), moderate (blue), and severe (red) clinical pain intensity.

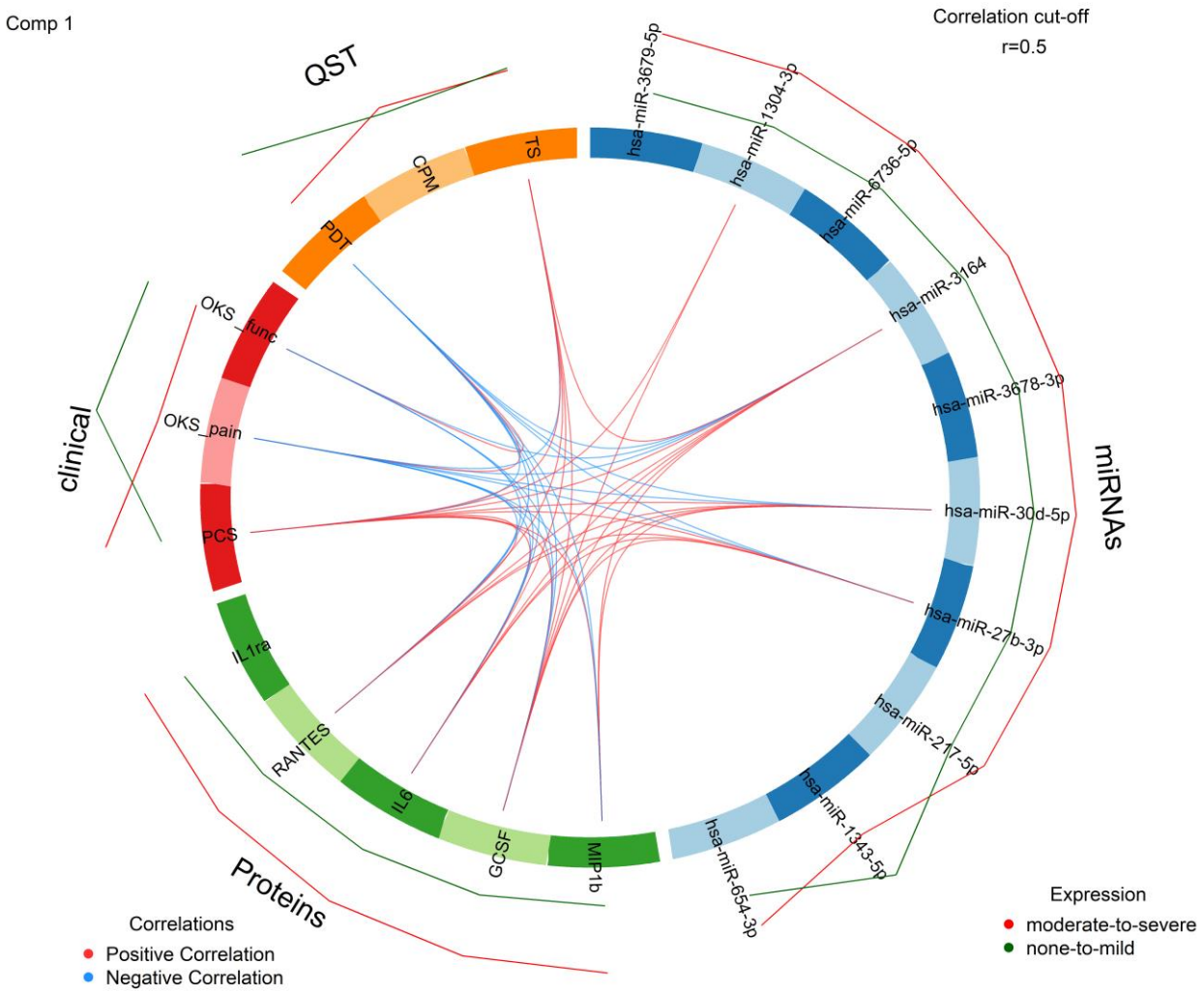
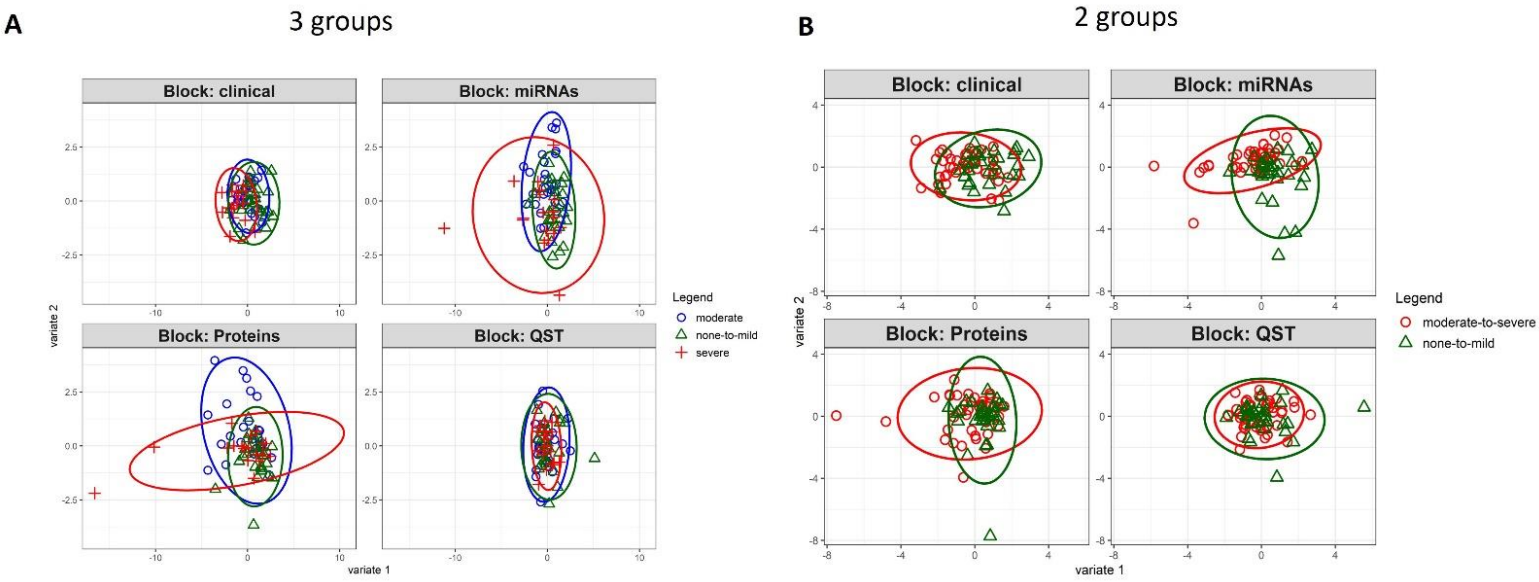


FIGURE 2. Circos plot from multiblock sPLS-DA for component 1 in 2 groups separation. The plots represent the correlations greater than 0.5 between variables selected for all parameters. The internal connecting lines show the positive (red) and negative (blue) inter-correlations. The outer lines show the expression levels of each variable in each sample group, none-to-mild (dark green), moderate-to-severe (red) clinical pain intensity.

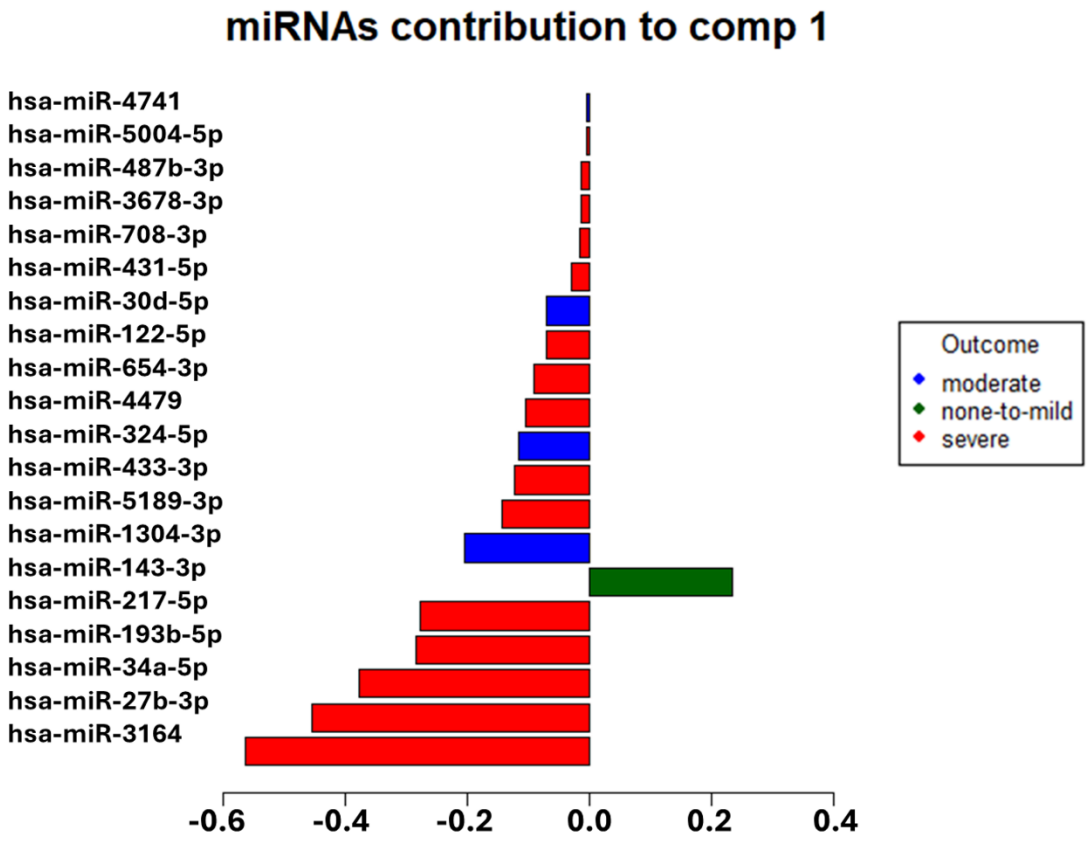
New supplementary materials:

S1. Principal component Analysis (PCA) sample plot from multiblock sPLS-DA. The samples are plotted based on their scores on 2 components (variate 1 and variate 2) for each data set. Samples are colored by pain subtype. For 3 groups none-to-mild (dark green triangles), moderate (blue circles), and severe (red crosses). For 2 groups none-to-mild (dark green triangles) and severe (red crosses). Ovals depict the 95% confidence intervals.

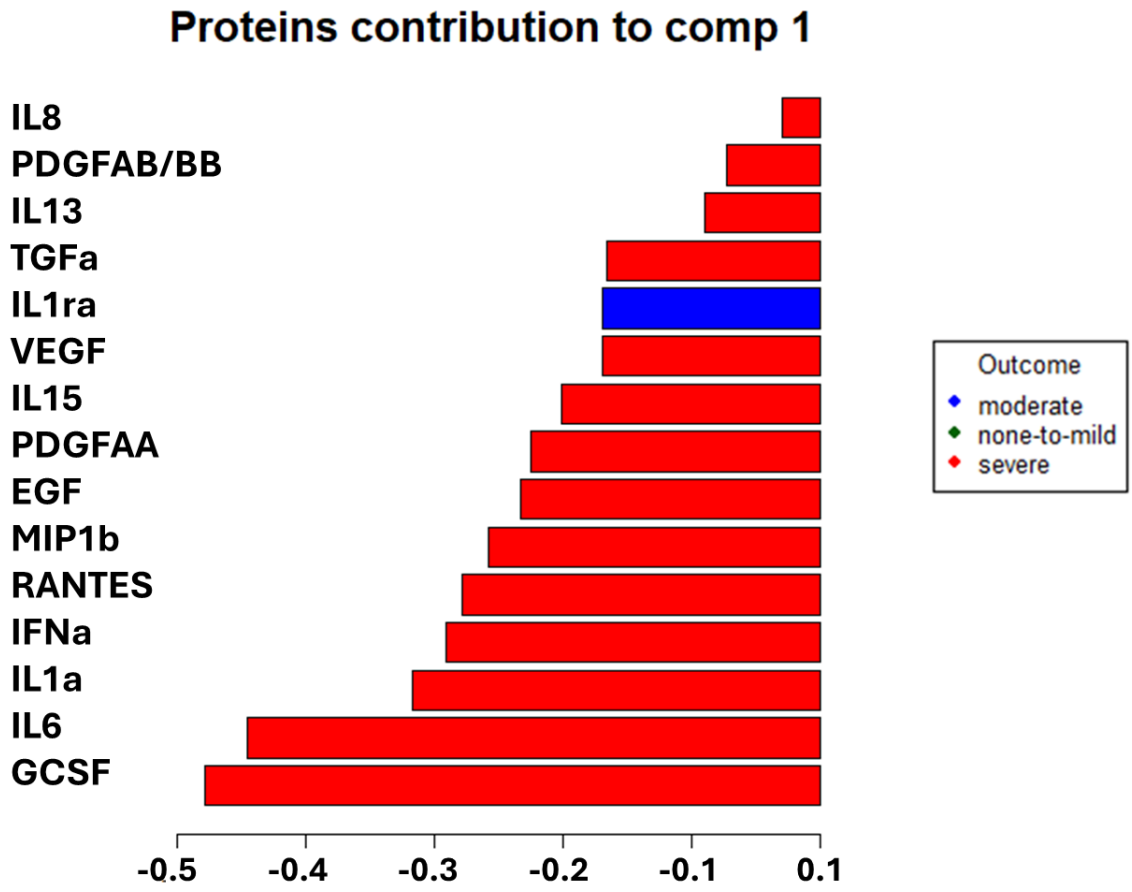


S2. Loading plot for miRNAs contribution for component 1 in 3 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors

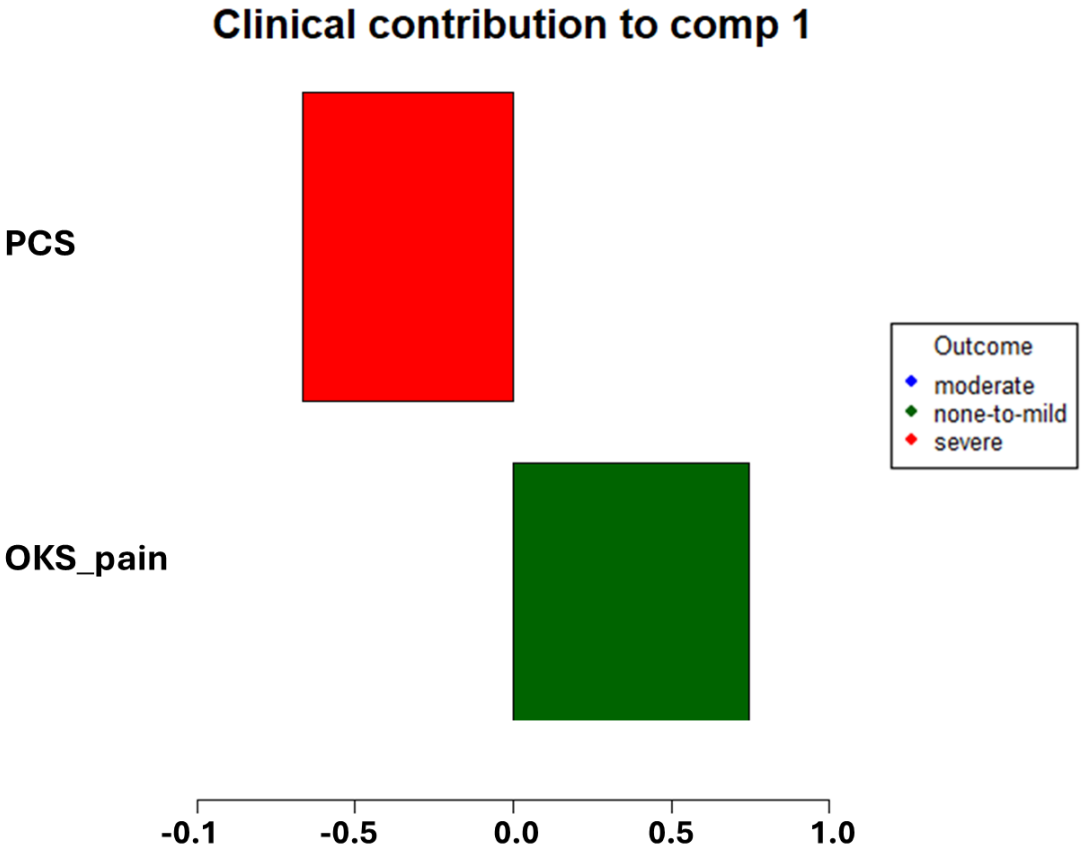
indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate (blue), and severe (red).



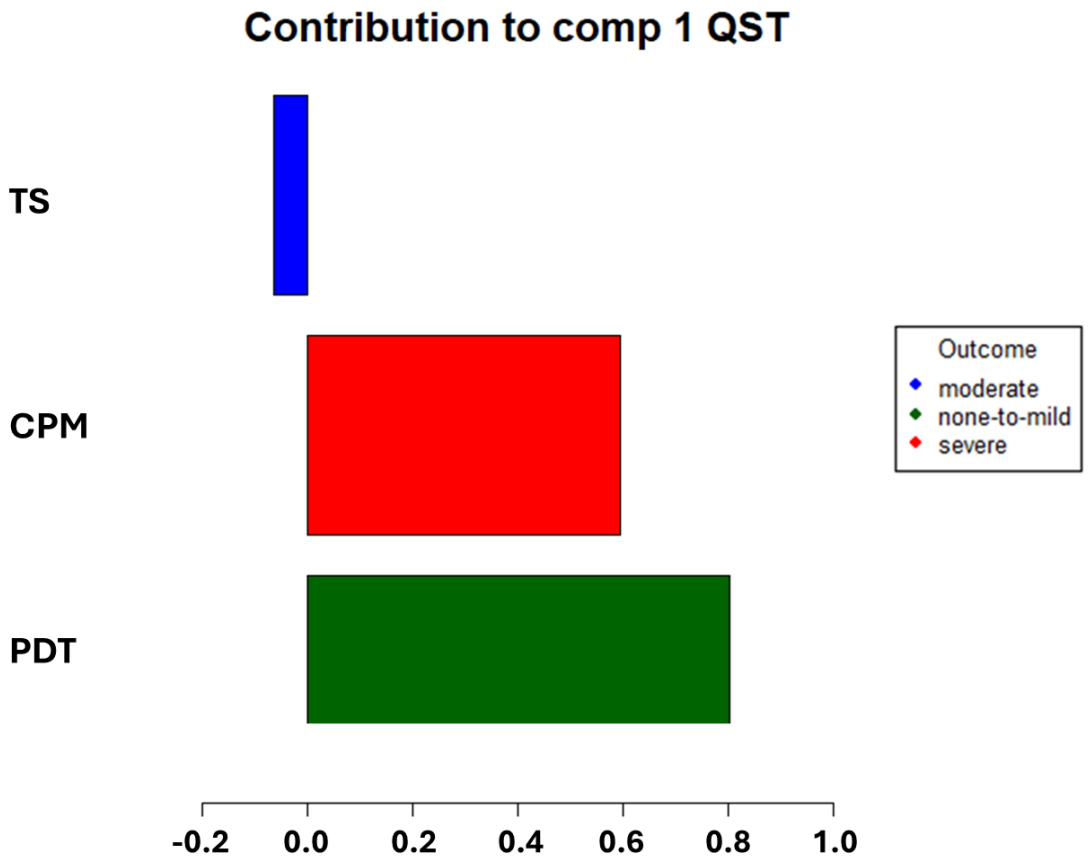
S3. Loading plot for proteins contribution for component 1 in 3 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate (blue), and severe (red).



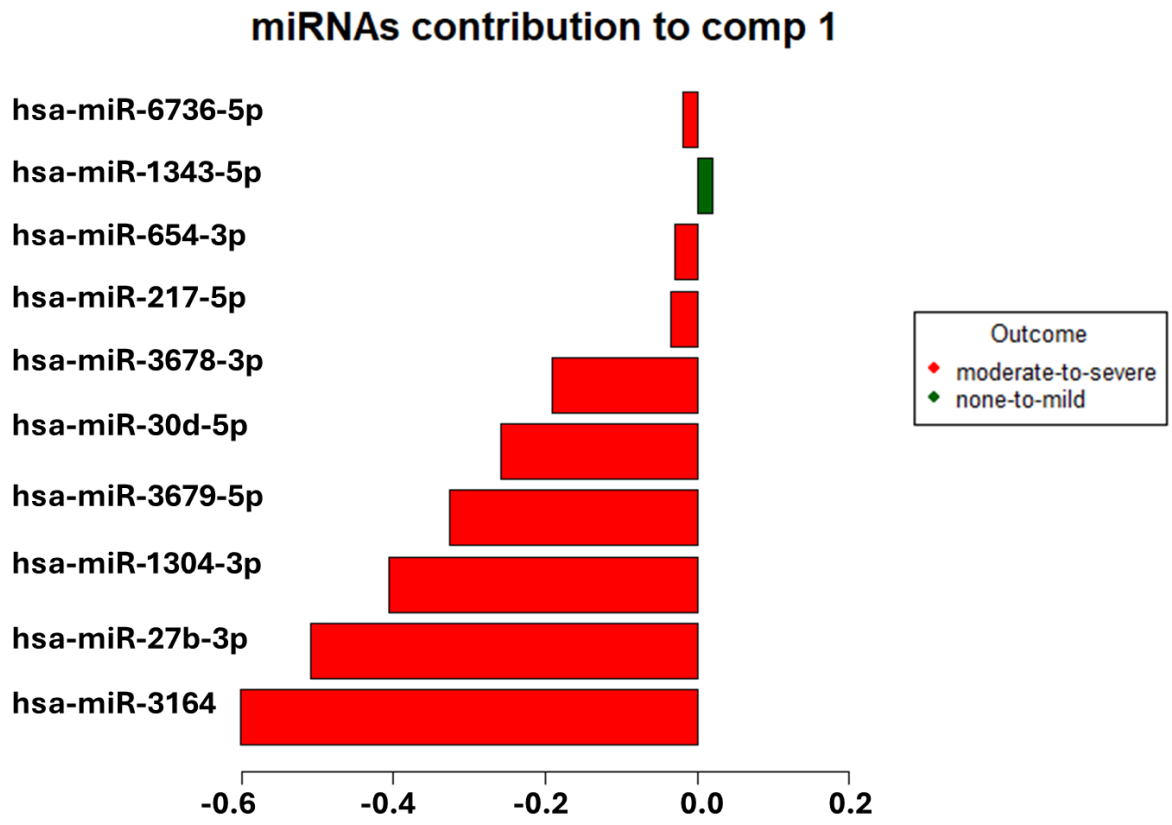
S4. Loading plot for clinical contribution for component 1 in 3 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate (blue), and severe (red).



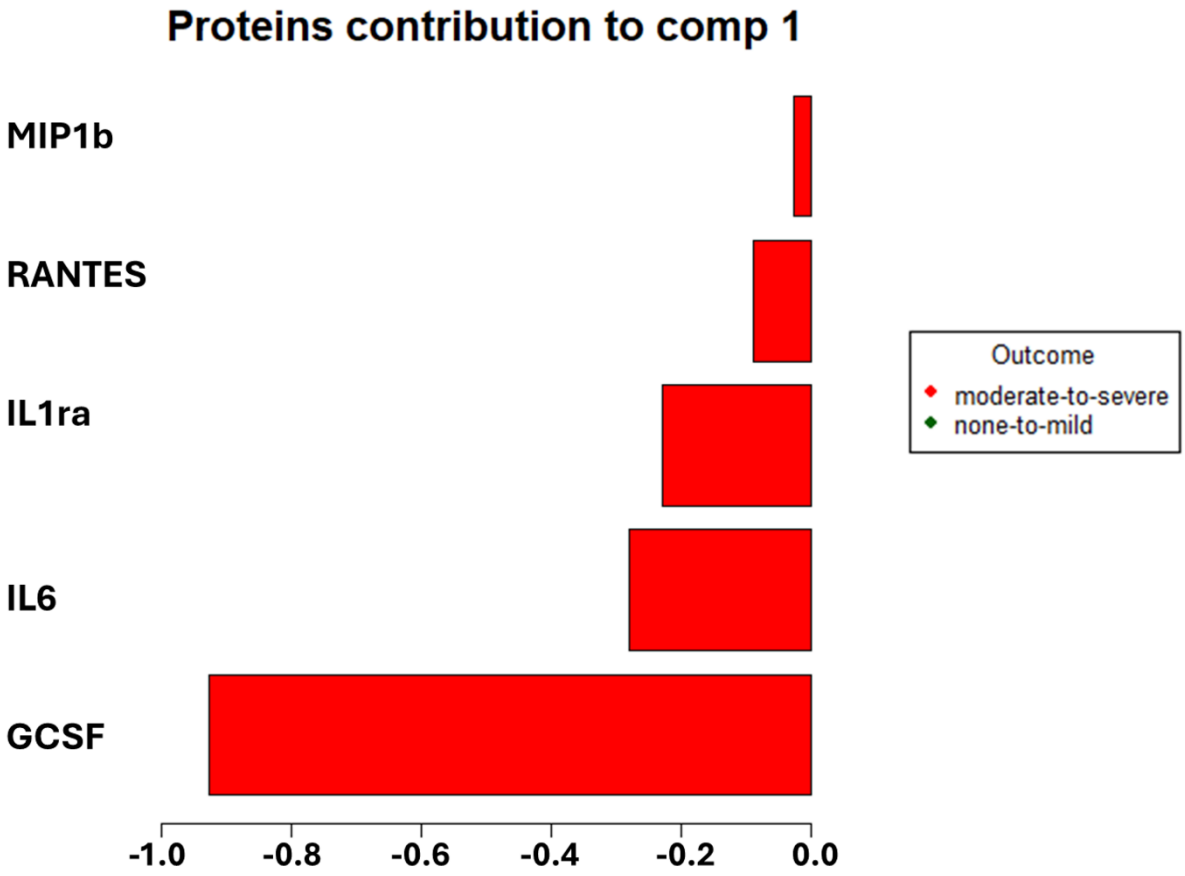
S5. Loading plot for QST contribution for component 1 in 3 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate (blue), and severe (red).



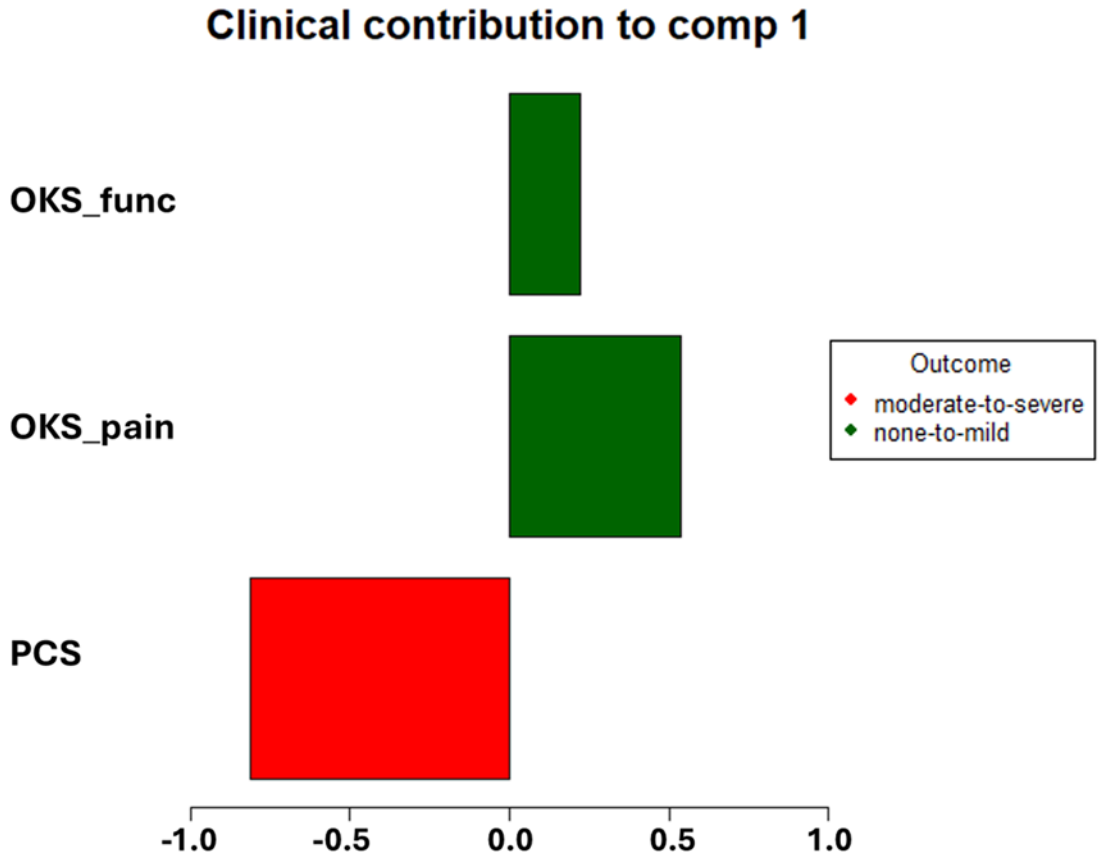
S6. Loading plot for miRNAs contribution for component 1 in 2 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate-to-severe (red).



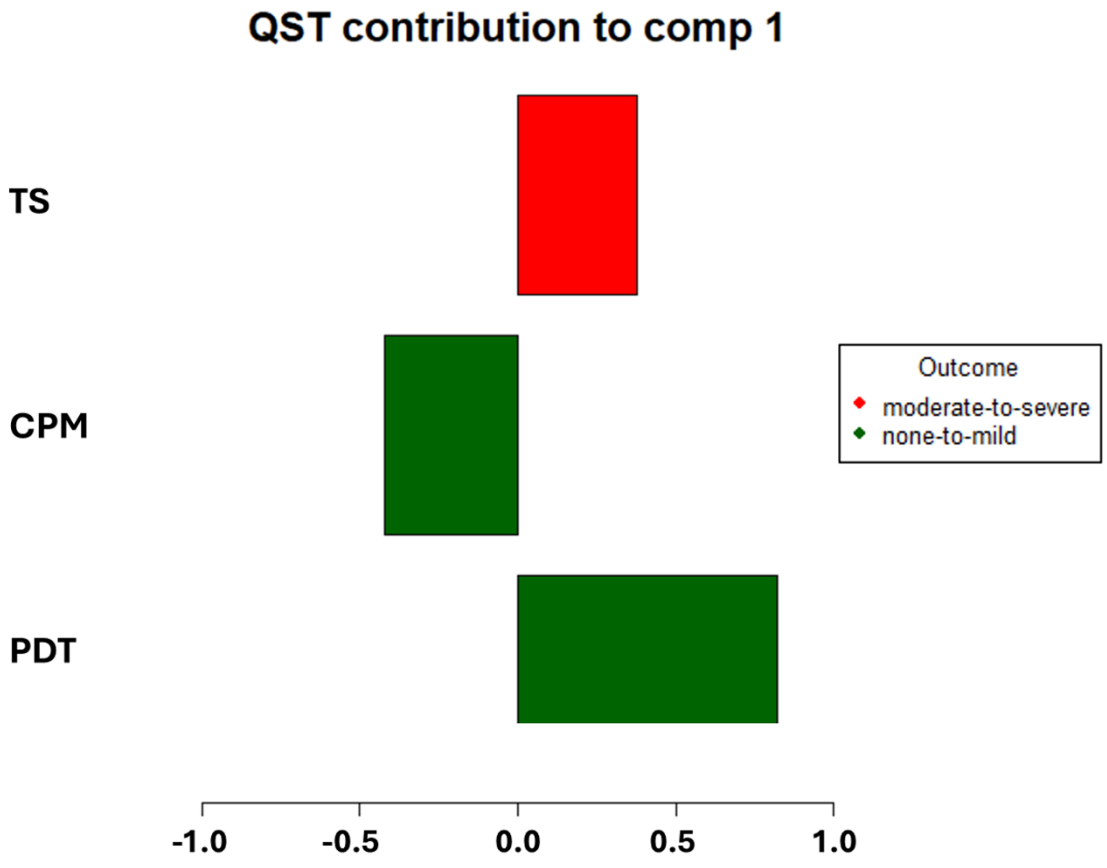
S7. Loading plot for proteins contribution for component 1 in 2 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate-to-severe (red).



S8. Loading plot for clinical contribution for component 1 in 2 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate-to-severe (red).



S9. Loading plot for QST contribution for component 1 in 2 groups model. The loading plot depicts of the most important variables ordered from bottom to top. The absolute value of the loading score indicates the importance or contribution of a variable to component 1. Colors indicate the class for which the average expression value is the highest for each feature none-to-mild (dark green), moderate-to-severe (red).



Comments 10-12: The responses to comments 10, 11, and 12 are all acceptable. The authors have integrated the feedback appropriately.

Conclusion: Giordano et al. have made significant improvements to their manuscript, "Pain Mechanistic Networks - Development using Supervised Multivariate Data Analysis and Implications for Chronic Pain."

Most of my comments have been addressed effectively, with only minor revisions needed to enhance figure clarity.

Reviewer #3

General comment: The authors have conducted a sufficient revision of the manuscript and have addressed my concerns as much as possible or have provided a sufficient explanation.
Authors response: We thank the reviewer for the dedication and hard work in reviewing our work.

Pain Mechanistic Networks – Development using Machine Learning Techniques and Implications for Chronic Pain

Chronic postoperative pain is present in approximately 20% of patients undergoing total knee arthroplasty (TKA). Studies indicate that pain mechanisms are associated with development and maintenance of chronic postoperative pain. The current study assessed pain sensitivity, inflammation, microRNAs, and psychological factors and combined these in a network to describe chronic postoperative pain.

This study involved 75 patients with and without chronic postoperative pain after TKA. Clinical pain intensity, Oxford Knee Score, and pain catastrophizing were assessed as clinical parameters. Quantitative Sensory Testing (QST) was assessed to evaluate pain sensitivity, microRNAs, and inflammatory markers were likewise analyzed. Supervised multivariate data analysis with 'Data Integration Analysis for Biomarker Discovery' using Latent cOmponents (DIABLO) was utilized to describe the chronic postoperative pain intensity. Two DIABLO models were constructed by dividing the patients into three groups or two defined by clinical pain intensities.

DIABLO model explained chronic postoperative pain and identified factors involved in pain mechanistic networks among assessments included in the analysis. Developing models of three or two patient groups using the assessments and the networks could explain 81% and 69% of the variability in clinical postoperative pain intensity. The reduction of the number of parameters stabilized the models and reduced the explanatory value to 69% and 51%.

This is the first study to use the DIABLO model for chronic postoperative pain and to demonstrate how different pain mechanisms form a pain mechanistic network. The complex model explained 81% while the less complex model explained 51% of the variability of clinical pain intensity.

Keywords: pain networks; microRNA; pain; targeted-omics; postoperative pain; functional outcomes; QST.

Pain Mechanistic Networks: The Development using Supervised Multivariate Data Analysis and Implications for Chronic Pain

Rocco Giordano [1,2], Lars Arendt-Nielsen [2,5,6,7], Maria Carla Gerra [3], Andreas Kappel [4], Svend Erik Østergaard [4], Camila Capriotti [2,3], Cristina Dallabona [3], Kristian Kjær-Staal Petersen [2,5].

Affiliations:

1. Department of Oral and Maxillofacial Surgery, Aalborg University Hospital, Aalborg, Denmark
2. Center for Neuroplasticity and Pain (CNAP), SMI®, Department of Health Science and Technology, Aalborg University, Aalborg, 9220, Denmark
3. Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parma, 43123, Italy.
4. Interdisciplinary Orthopedics, Department of Orthopedic Surgery, Aalborg University Hospital, Aalborg University Hospital, Aalborg, Denmark
5. Center for Mathematical Modeling of Knee Osteoarthritis (MathKOA), Department of Material and Production, Faculty of Engineering and Science, Aalborg University, Aalborg, Denmark.
6. Department of Gastroenterology & Hepatology, MechSense, Aalborg University Hospital, Aalborg, Denmark.
7. Steno Diabetes Center North Denmark, Clinical Institute, Aalborg University Hospital, Aalborg, Denmark

***Corresponding Author:**
Associate Professor Kristian Kjær-Staal Petersen, M.Sc., Ph.D., D.M.Sc.
Center for Neuroplasticity and Pain
Department of Health Science and Technology
Faculty of Medicine, Aalborg University
Selma Lagerløfs Vej 249, DK-9260 Gistrup, Denmark
Phone: +45 9940 7529, E-mail: KKP@HST.AAU.DK

Number of Pages: 20
Number of Figures: 3
Number of Tables: 2

Data related to the present work will be available upon reasonable request to the corresponding author.

Introduction

Chronic postoperative pain is a complex problem [41,66] that occurs in approximately 20% of patients undergoing total knee arthroplasty (TKA) [11]. Studies suggest that pain sensitivity, inflammation, microRNAs, and psychological factors are associated with clinical pain [53]. A recent commentary called for the integration of these parameters into models to understand the factors driving the clinical pain intensity [55].

Quantitative sensory testing (QST) is a neurological assessment and pressure pain thresholds (PPTs), temporal summation of pain (TSP), and conditioned pain modulation (CPM) are believed to reflect neural activity in the peripheral, dorsal horn, and descending pain inhibitory systems [17,58,76]. Studies suggest that pain sensitivity measures are associated with chronic postoperative pain after TKA but that the explanatory value of QST is limited to explain approximately 20% of the postoperative pain variability [41,51,52]. Animal research suggests that pain sensitivity arises due to pro-inflammatory cytokines [60,63]; but this has not been confirmed in human studies.

Pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-6 have been found upregulated in patients with osteoarthritis and associated with increased clinical pain [20,23]. MicroRNAs (miRNAs) are involved in post-transcriptional gene expression regulation and are highly stable in body fluids [74]. In other fields of research, miRNAs have been linked to chronic stress and the development and functionality of the brain [37], but have still not been extensively applied in the field of pain. A complex combinatorial code exists whereby a single miRNA may regulate several targets, and the same targets can be co-regulated by different miRNAs [21]. Thus, the functionalities of miRNAs are plenty. Interestingly, e.g., miRNA-146a is believed to regulate the expression of IL-1β and TNF-α [15,49], suggesting a link between inflammation and miRNAs. Psychological factors have emerged to be directly or indirectly associated with chronic pain in osteoarthritis and include a range of factors such as anxiety, depression, and pain catastrophizing [18]. A recent study assessed 100 patients with chronic osteoarthritis pain [34] and demonstrated that pain catastrophizing was a strong predictor for clinical pain and that the contribution was larger than the assessed QST parameters – yet both pain catastrophizing and QST were independently important for the explanation of clinical pain.

In summary, pain sensitivity, inflammation, miRNAs, and psychological factors are considered important contributors to the manifestation of chronic pain. Evidence suggests that these factors might interact and work in networks, but this has not been studied in detail. Sporadic preliminary evidence suggests that particular pain networks might exist and that, e.g., pain sensitivity might be associated with pain catastrophizing in patients with chronic low back pain [14]. Further, animal research suggests that inflammation can sensitize the peripheral and

central nervous system and lead to pain sensitivity [61,63] but the interaction between QST and inflammation has not been confirmed in humans. An in-depth understanding of this would be novel and push the scientific field forward.

The aim of the current study was to include a variety of factors assumed to be associated to clinical pain intensity 5-years after TKA surgery and to utilize a supervised machine learning technique to identify if mechanisms could form pain mechanistic networks and if these pain mechanistic networks could explain chronic postoperative pain.

Materials and methods

Patients' cohort

The current work is a secondary analysis of Skrejborg et al., 2021 [66]. The report by Skrejborg et al., 2019 [66] focused on the differences of clinical parameters of patients with and without chronic postoperative pain 5 years after TKA. The current work utilizes some of the same parameters and adds a more in-depth analysis of inflammatory markers and miRNA with the aim of investigating the multimodal aspects of chronic pain and the potential to identify pain mechanistic networks.

In this study, 80 patients who underwent TKA in 2011 in five different Northern Denmark hospitals were enrolled for a subsequent five-year observational follow-up [66]. Exclusion criteria set for the study included any additional surgical interventions between 2011 and the follow-up period, inadequate proficiency in Danish, a medical history inclusive of neurological or psychiatric disorders, inability to participate effectively in the study, or documented substance abuse history. At the follow-up visit, conducted at the Orthopedic Outpatient Clinic of Aalborg University Hospital, Denmark, QST evaluation was performed, and 5 ml of blood was extracted via forearm venipuncture from each patient. Subsequently, plasma was isolated using centrifugation at 2000g for a duration of 15 minutes and preserved at a temperature of -80°C until investigation of circulating biomarkers. Expert orthopedic surgeons conducted thorough evaluations to exclude participants with indications of periprosthetic joint infection, aseptic loosening, or mechanical complications necessitating revision surgery. The study was approved by the local ethics committee (N-20170072) and by the Danish Data Protection Agency and followed the rules of the Declaration of Helsinki. All patients signed an informed consent before enrollment.

Questionnaires

Before the follow-up visit, patients were asked to rate the average knee pain intensity within the last 24-hours on a 10 cm visual analog scale (VAS), where 0 represented no pain, and 10 represented the worst pain imaginable. Additionally, patients underwent evaluations using the Oxford Knee Score (OKS)[48] and the Pain Catastrophizing Scale (PCS) [69], as detailed below.

The PCS is a tool for assessing pain-related catastrophizing thoughts, comprising a 13-item questionnaire that focuses on pain-related thoughts and emotions. Each item is rated on a 5-point Likert scale, from 0 (not at all) to 4 (all the time). The total PCS score, ranging from 0 to 52, is calculated by summing the responses to all items. A score above 30 on the PCS is considered a clinical threshold for indicating significant pain catastrophizing thoughts [69].

The OKS, specifically designed for evaluating knee pain and function before and after knee surgery consists of 12 items [50]. It assesses knee pain (OKS-pain; 7 out of 12 items) and function (OKS-function; 5 out of 12 items) in knees affected by OA [33,48]. The scores for each subscale are standardized, with this study applying a range from 0 (worst possible outcome) to 100 (best possible outcome) for standardization.

Quantitative Sensory Testing (QST)

A computer-controlled cuff pressure algometer (Cortex Technology and Aalborg University, Denmark) with two 13cm tourniquet cuffs (VBM Medical, Germany) and an electronic visual analog scale (Aalborg University, Denmark) was used to record deep-tissue pain sensitivity. The cuff was placed at the level of the head of the gastrocnemius muscle on the leg most affected by OA. The electronic visual analog scale was anchored at 0; “no pain” and 10; “maximum pain”. Test–retest reliability of computer-controlled cuff algometry for assessment of pain threshold, TSP, and CPM have been demonstrated in pain-free subjects [23,24,34,35,70] and individuals with chronic pain [69] to be good-to-excellent.

Cuff pressure pain and tolerance thresholds

The pressure was increased at a rate of 1 kPa/s, and the patient was instructed to continuously rate their pain on the electronic visual analog scale until reaching their tolerance. The patient was equipped with a stop button to press when reaching their tolerance. The cuff pressure pain threshold (cPPT) was defined as the pressure at which the electronic visual analog scale exceeds 1 cm, and the pain tolerance threshold when the patient pressed the stop button.

Temporal summation of pain

Ten repeated mechanical pressure stimuli will be delivered at 0.5 Hz (1-s stimuli duration and 1-s interstimulus intervals). During the 10 repeated stimuli, the subjects will continuously rate the pain intensity on the electronic VAS. TSP was defined as the difference between the electronic visual analog scale scores of the tenth and first stimuli.

Conditioned modulation of pain

An additional tourniquet cuff was fitted around the contralateral leg and a painful tonic stimulus was applied at 70% of the cPTT recorded previously. Simultaneously, the other cuff applied a gradually increasing pressure of 1kPa/s rated continuously on the electronic visual analog scale until tolerance was reached, indicated by the patient pressing the stop button. The CPM effect will be defined as the difference between the PPT during and before the conditioned pain with a positive value denoting functional CPM.

miRNA Analysis

RNA was isolated from 200 µl of plasma using the miRNeasy Serum/Plasma Kit (QIAGEN) according to the manufacturer's instructions with an elution volume of 14 µl. The library preparation was done using the QIAseq miRNA Library Kit (QIAGEN). A total of 5 µl total RNA was converted into miRNA NGS libraries. After adapter ligation, UMIs were introduced in the reverse transcription step. The cDNA was amplified using PCR (22 cycles), and during the PCR, indices were added. After PCR, the samples were purified. Library preparation was quality controlled using capillary electrophoresis (Fragment Analyzer HS NGS Fragment Kit (1-6000bp)). Based on the quality of the inserts and the concentration measurements, the libraries were pooled in equimolar ratios. The library pool(s) were quantified using qPCR. The library pool(s) were then sequenced on a NextSeq (Illumina Inc.) sequencing instrument according to the manufacturer's instructions (1x75, 2x10). Raw data was de-multiplexed, and FASTQ files for each sample were generated using the bcl2fastq2 software (Illumina Inc.). All primary analysis was carried out using CLC Genomics Server 23.0.5. The workflow "QIAseq miRNA Quantification" of CLC Genomics Server with standard parameters was used to map the reads to miRBase version 22. In short, the reads were processed by (1) trimming of the common sequence, UMI, and adapters, and (2) filtering of reads with a length < 15 nt or length > 55 nt. They were then deduplicated using their UMI. Reads were grouped into UMI groups when they (1) started at the same position based on the end of the read to which the UMI was ligated (i.e., Read2 for paired data), (2) were from the same strand, and (3) had identical UMIs. Groups that contained only one read (singletons) were merged into non-singleton groups if the singleton's UMI could be converted to a UMI of a non-singleton group by introducing an SNP (the biggest group was chosen).

Protein Analysis

Analysis of proteins consisted of the investigations of a predefined list of forty-four analytes which included interleukins, interferons, chemokines, and growth factors, using the Luminex assay that allows simultaneous assessment of multiple human cytokines' levels in a single sample. In this study, the Human XL Cytokine Magnetic 44-plex Luminex® assay (R&D Systems, USA) was used (full list in supplementary). Plasma samples were slowly defrosted at 4°C and centrifuged at 16,000 x g for 4 minutes immediately before the analyses, which included two steps. First, 50 µl of 2-fold diluted plasma in duplicates for each sample were incubated on a 96-well plate with 50 µl magnetic capture beads containing analyte-specific antibodies. The plate was incubated overnight in the cold chamber on a horizontal orbital microplate shaker set at 900 ± 50 rpm. The second step included the washing processes with a magnetic device designed to accommodate the microplate; after that, three Buffer's washes were alternated with Biotin-Antibody Cocktail and Streptavidin-PE incubations according to the manufacturer's protocol. Lastly, 100 µl of Wash Buffer was added to each well to read the plate in the MAGPIX Luminex system (Luminex, Austin, Texas, United States).

Statistical analysis

Definition of variables domain.

In this study data obtained from each assessment were divided into domains. One domain represented by QST assessments, includes pain detection threshold (PDT), conditioned pain modulation (CPM), and temporal summation (TS). The second domain was represented by clinical assessments including, questionnaire results obtained from Oxford knee score for pain (OKSpain) and function (OKSfunction), and Pain catastrophizing (PCS). Moreover, two domains included biological data, specifically miRNA expression levels and targeted protein quantifications (inflammatory markers) were developed.

DIABLO analysis

Before analysis, data from each domain - miRNAs, proteins (inflammatory mediators), clinical outcomes, and QST evaluations - underwent a preprocessing phase. This included scaling steps to ensure comparability across different data types and variables with near-zero variance were identified and removed to improve the model's performance and interpretability.

In this study, biological, clinical, and QST assessment data integration was conducted using the Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) framework. The DIABLO tool is a statistical

method that aims to explain an outcome (clinical pain in the current study) using multiple datasets by identifying relationships and thereby explaining a complex system (clinical pain in the current analysis) [65]. DIABLO implements several approaches, namely Multiblock Partial Least Squares [43], generalized canonical correlation analysis (GCCA) [71], and Multi-Omics Factor Analysis (MOFA) [7], and uses these in a supervised classification framework by employing singular value decomposition to select and project correlated variables from multiple datasets into a lower-dimensional space. This method can be used to explain relationships between variables, which helps identify key correlated parameters across different levels of data [65].

In the current study, we developed two DIABLO models aiming to explain the categorization of patients into three groups: none-to-mild (VAS 0-2), moderate (VAS 3-6), and severe (VAS 7-10) clinical pain intensity [12]. To investigate the robustness of the DIABLO analysis, another DIABLO model with two groups based on clinical pain was developed with groups defined as none-to-mild (VAS 0-3) and moderate-to-severe (VAS 4-10) clinical pain intensities [29]. The analysis was run with a design matrix of 0.5 value (from 0 to 1) to integrate the different datasets. This value indicates a moderate expected correlation between the datasets, indicating an assumption of partial interrelation among the various dataset layers. This was followed by the construction of a sparse Partial Least Squares Discriminant Analysis (sPLS-DA) model, focusing on three components and actively excluding predictors with minimal variance. The initial model's performance was assessed using a cross-validation approach, specifically M-fold cross-validation repeated several times. This process aimed to evaluate the model's predictive accuracy and to determine the benefit of incorporating additional components. A tuning phase followed, where the optimal number of variables to retain in each component was determined. This step involved adjusting specific parameters and selecting a range of variables for each data type. After fine-tuning, the model incorporated the best combination of variables. Various visualizations were then generated, including plots of individuals for components included, Circos plots for cross-correlations, and loading plots. A visualization of component 1 was chosen as this includes variables identified by the model that explains the most significant portion of the variation in the data. The Circos plot was configured with a correlation threshold of 0.5 to define the cross-correlation between selected variables by the model, effectively identifying the pain mechanistic network. Loading plots were utilized to analyze variable relationships with component 1, classifying associations based on loading values into weak (-0.3 to 0.3), medium (0.3 to 0.7 or -0.3 to -0.7), and strong (>0.7 or <-0.7). The visualization was used for the interpretation of the discriminative features within the different data domains and for understanding the relationships between different data types. Data integration and DIABLO framework a component of the mixOmics package (mixOmics V. 6.26.0) [42,57] and R studio (V. 2023.12.0) were used.

After discrimination, the variables reported in the circus plot were used in linear regression models to explain clinical pain intensity. To validate the models, we conducted a Leave-One-Out Cross-Validation (LOOCV) and assessed the Root Mean Square Error (RMSE) as a measure of the model's quality and validity [43,71]. The RMSE provided a quantitative assessment of the accuracy with which the model predicted patient pain levels, thereby confirming its reliability (with lower values indicating a more stable model). Data analysis was performed in in R studio (V 4.3.2.) and SPSS software V.29 (IBM, Armonk, New York, USA) and $p<0.05$ was considered significant.

Results

Demographics

A total of 75 patients completed all assessments and were included in the analysis. Most patients had one or more comorbidity (92%), with the most prevalent comorbidities being osteoarthritis in the contralateral knee: 76.0% osteoarthritis in more than 2 joints: 20.0%, arthritis: 9.33%, diabetes: 13.33%, and a history of cancer: 13.33%, see Skrejborg et al., 2019 [66]for a full analysis of the effect of comorbidities on clinical pain intensity 5-years after TKA surgery. Demographics related to the patients can be found in Table 1.

MicroRNAs and inflammatory markers analyses

In the investigation of miRNAs, the mapping rates varied from 7.72% to 49.21%, indicating a low to moderate matching to sequence references, which was sufficient for all downstream analyses. Analysis of reads mapped with miRBase revealed the presence of 2632 miRNA species in the evaluated samples. MiRNAs mapped that were present in less than 50 % of the patients were excluded due to their contribution to the variance, leaving 826 miRNAs included in the subsequent analysis. Similarly, for protein analysis, markers contributing zero to the variance were excluded, resulting in 39 markers being incorporated into the final analysis.

Data integration of pain mechanisms and development of pain mechanistic networks

Four models were developed using DIABLO and linear regression with backward elimination. All models were different in number of predictors, explanation value (R^2-value), and robustness (RMSE). An overview of all the models is found in table 2, and detailed description of the model developments and findings are found below.

The integration of patients' values for every data block (miRNAs, proteins, QST, clinical outcomes) showed a grouping tendency based on their values for component 1 and were classified into either three groups based on their clinical pain intensity (none-to-mild, moderate, and severe pain intensity), or in 2 groups (none-to-mild, and

moderate-severe pain intensity). These clusters were not distinctly separated, indicating a degree of overlap, or intermixing among the groups (Figure S1 A-B).

Models based on 3 groups

With this list of dataset blocks, the tuning of the Partial Least Squares Discriminant Analysis (sPLS-DA) model highlighted 40 variables related to pain categorization (none-to-mild, moderate, and severe) as the best combination of variables for the model. The Circos plot illustrated the cross-correlations between the 40 variables included in the model and how these variables are correlated with each other (Pearson's correlation coefficients > 0.5 for identification of pain mechanistic networks, see Figure 1). Furthermore, the loadings plot revealed distinct contributions of various features to the first principal components. The observed loading values for these variables varied significantly across the blocks, miRNAs (Figure S2), proteins (Figure S3), clinical (Figure S4), and QST Figure S5) indicating differential contributions to the component structure. When investigating 3 groups miRNAs loading values suggested a moderate to strong negative (Figure S2) association with the first component. Notably, variables within this block showed a predominant linkage to the severe clinical pain intensity group outcome. The protein variables presented a narrower range of loading values indicating a weaker negative association with component 1 (Figure S3). The variables in this block were also primarily associated with the severe pain intensity group outcome, though the strength of this association was less pronounced than in miRNAs' block. Clinical variables demonstrated a unique distribution of loading values, with a strong negative association for PCS in the severe pain group and a strong positive association in the non-to-mild pain group (Figure S4). QST variables showed a strong positive (Figure S5) correlation between CPM and PDT in the severe pain and none-to-mild pain groups, respectively. A weak (Figure S5) association was observed for TS in the moderate pain group.

****

Figure 1 around here

****

Models based on 2 groups

The tuning of the Partial Least Squares Discriminant Analysis (sPLS-DA) model highlighted 21 variables related to pain categorization (none-to-mild, and moderate-to-severe) as the best combination of variables for the model.

The Circos plot represented the cross-correlations between the 21 variables included in the model and reported variables intercorrelations for identification of pain mechanistic networks (Pearson's correlation coefficients > 0.5, see Figure 2). Moreover, single variables included in the model showed a relative alteration between the 2 groups identified (outer ring in Figure 2).

****

Figure 2 around here

****

When evaluating 2 groups MiRNA loading values suggested a moderate to strong negative (Figure S6) association with the first component with a predominant linkage to the moderate-severe pain intensity group outcome. The protein variables presented a narrower range of loading values indicating a weaker negative association with component 1, except for GCSF which showed a strong association with the investigated component (Figure S7). Clinical variables demonstrated a unique distribution of loading values, with a strong negative association for PCS in the moderate-severe pain and a weak to medium positive association in the none-to-mild pain group (Figure S8). QST variables showed a medium positive (Figure S9) contribution for CPM in the moderate-severe pain group, a strong contribution of PDT in none-to-mild pain groups, and a medium contribution was observed for TS in the none-to-mild pain group.

Linear regression models

The linear regression analysis incorporated the 40 variables from the DIABLO analysis and investigated their contribution to clinical pain intensity (Table 3). The model demonstrated an explanatory power, accounting for 81% of the variance in clinical pain intensity ($R^2 = 0.81$, $F = 3.369$, $p < 0.0001$). This indicates that the model explains 81% of the variability in clinical pain from the current cohort. Despite the feature selection, the validation model though LOOCV resulted in a relatively high Root Mean Squared Error (RMSE) of 3.79 (0-∞). Given this outcome, to further refine the model, a linear regression with backward elimination was constructed. The second model narrowed down the variables to 12 which demonstrated the most substantial impact on the model's performance holding an R2 of 0.69 ($R^2 = 0.0,69$, $F = 11.294$), $p < 0.0001$) (Table 3). Validation with LOOCV showed a significant improvement with a RMSE decreased to 1.86.

Furthermore, linear regressions were constructed on 21 variables chosen from the DIABLO approach for groups of patients divided based on the clinical cut-off (VAS 3) (Table 4). Model 1 accounted for 63% of the variance in pain perception ($R^2 = 0.63$, $F = 4.344$, $p < 0.0001$) (Table 4), and cross-validation reported an RMSE of 2.15.

Applying a backward elimination model to model 2, reduced the number of parameters to 5, which could explain 51% of the variance in clinical pain intensity ($R^2 = 0.51$, $F = 14.461$, $p < 0.0001$) and cross-validation reported an RMSE of 1.88.

Discussion

This is the first study to utilize DIABLO framework to identify factors that form pain mechanistic networks that can explain chronic postoperative pain. The current study demonstrated that measures of pain sensitivity, psychological factors, microRNAs, and inflammation levels, are all linked in large pain mechanistic networks. Developing models of either three or two groups using the assessments and the pain mechanistic networks could explain 81% and 69% of the variability in clinical postoperative pain intensity. Reducing the number of parameters stabilized the models and reduced the explanatory value to 69% and 51%.

Utilizing quantitative sensory testing and psychological factors to explain clinical pain intensity

QST has been utilized in the assessment of chronic musculoskeletal pain for a decade and although QST findings are correlated to clinical pain intensities [3,4,6,46], the explanatory value of QST for clinical pain intensities remains low for patients with osteoarthritis [54]. Similarly, several studies have found that levels of depression, anxiety, or pain catastrophizing thoughts are associated with clinical pain intensities [13,19,41], but the explanatory value of these psychological factors for clinical pain intensity remains low. Inflammation can be assessed in multiple forms in osteoarthritis and measures such as C-reactive protein (CRP) [64], synovitis [9,36], or more recently using a broader spectrum of proteins [27]. Conclusively inflammatory markers might be associated with clinical pain intensities in patients with osteoarthritis, but the explanatory value for clinical pain remains low.

Recent study assessed QST, psychological factors, and quality of life finding that combination of these three domains explained 36.2% of clinical pain intensity in patients with osteoarthritis [34]. Additionally, another study assessed QST, the PCS, and joint magnetic resonance imaging (MRI) to identify synovitis and found that this could explain 65.3% of the clinical pain intensity in patients with and without chronic postoperative pain after TKA [41]. These data do indicate that the integration of multiple domains might increase our understanding of why patients with chronic pain experience different degrees of clinical pain intensities. The current study builds upon the above-mentioned research findings but adds to the literature by utilizing the DIABLO approach to explain clinical pain intensity 5-years after TKA surgery and is able to demonstrate that assessments of QST, clinical factors, a range of proteins, and miRNAs can explain 81% of the variability in clinical pain intensity.

The use of inflammatory markers and miRNA in understanding postoperative pain

Pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) have been found upregulated in patients with osteoarthritis pain and associated with increased clinical pain [20,23]. Recent studies have suggested that inflammatory markers can form networks that may help identify patients at high risk of developing chronic postoperative pain after total knee arthroplasty (TKA) [26]. Additionally, these networks have been shown to possibly underscore the severity of neuropathy and neuropathic pain in patients with diabetic polyneuropathy[8]. Concerning pain, miRNAs are believed to regulate the expression of several inflammatory markers [15,49] and pre-clinical data links these with a sensitization of the nervous system [63]. Some studies have seen an alteration in miRNAs following acute experimental pain [16,24,25,28] and preclinical and clinical studies have found that preoperative miRNAs can be linked to chronic postoperative pain [30,44,45,70]. The current study identifies some miRNAs (miRNA-433,-27b,-30d,-122) that have previously been associated with the expression of receptors involved in pain pathways or found to be biomarkers for pathological conditions characterized by pain [1,22,40,73]. Specifically, an increase in miRNA-34a-5p has been demonstrated in patients with painful migraines [1,22]. Moreover, miRNA-30d has been shown to regulate GABA in chemotherapy-induced peripheral neuropathy [73]. MiRNA-122 has been shown in animal models to regulate neuropathic pain [72]. Additionally, miRNA-27b has been previously associated with pain in osteoarthritis, with high levels shown in OA patients, and its regulation has been suggested to alleviate pain [78]. Although preliminary, this suggests that certain miRNAs might be coding for pain or nociception and potentially these are not specific to a certain disease but rather a marker for pain or nociception, but this is to be explored further in the future.

The development of pain mechanistic networks

Chronic pain, including chronic postoperative pain, is considered a multifactorial clinical problem [54] and factors such as socioeconomic status [39], sex [38], education [77], and more biological-oriented factors such as inflammation [27], sensitization of central pain pathways [5], and psychological factors [19] have been demonstrated to impact chronic pain. These factors can be viewed as different domains, and these domains can likely interact with each other, but this has not been studied in much detail. Studies have suggested that poor quality of sleep might modulate levels of anxiety, depression, and pain catastrophizing [6] and that can be associated with increased pain sensitivity [13,35,68], which suggests an interaction between the psychological domain and the nociceptive domain. This is further supported by a recent study on patients with chronic low

back pain, in which increased levels of pain catastrophizing were associated with impairment of CPM [8], suggesting a link between the two domains. Animal research indicates that inflammation can sensitize the peripheral and central nervous system and lead to pain sensitivity [59,62], suggesting a link between the immune system domain and the nociceptive domain. This link between inflammation and QST findings has not been confirmed in humans, although inflammation is widely studied in humans and the current study is therefore the first to demonstrate this association in humans. Potential explanation for this link between gene regulation, the inflammation process, and pain mechanism can be driven by epigenetic modifications [32]. This regulatory mechanism influences immune responses without altering the DNA sequence, showing the interplay between genetics and environmental factors [32,47]. Among epigenetic modifications, miRNAs have been shown in animal models and humans to be highly involved in the expression of specific inflammatory markers [10,31,67,75]. Furthermore, there is increasing evidence exploring the role of these epigenetic regulators in humans, particularly their association with specific pain conditions such as musculoskeletal pain [31], and neuropathic pain [56]and in general in chronic pain [47]. The current analysis is the first to demonstrate how four domains of chronic pain, namely pain sensitivity, inflammation, epigenetics, and psychological factors interact with one another, and these data demonstrate the importance of viewing chronic pain as a multifactorial problem. Future studies are encouraged to replicate these findings and to advance our understanding of chronic pain by adding other important factors for chronic pain.

Limitations

The current study is the first to utilize the DIABLO model to explain clinical pain intensity 5-years after TKA surgery and these findings must be replicated in an external cohort of patients. Comorbidities have been found associated with increased clinical pain [66] and the association between comorbidities and clinical pain should be further investigated. Medication intake, especially anti-inflammatory drugs, plays a role in the regulation of inflammatory markers and potentially the miRNA expression. The use of topical nonsteroidal anti-inflammatory drugs (NSAIDs) is recommended as a first-line treatment and oral non-selective and COX-2 inhibitor NSAIDs are recommended as 2-stage treatments by international clinical guidelines[2], and it is therefore likely that most patients use NSAIDs regularly. The current study did not assess the use of medication, which is a limiting factor to the study and should be considered when interpreting the data. The exploratory nature of the study did not allow predetermination of a specific sample size calculation, and this limitation should be taken into account when interpreting the findings. Post-hoc sample size calculation for the multiple linear regression models using a power of 80%, a significant level of 0.05, and an effect size of medium-to-large suggests that 58 patients are

needed for the use of 5 independent parameters and 81 patients are needed for the use of 12 independent parameters. This post hoc analysis suggests that two linear regression models that applied backward elimination are adequately powered. A sample size calculation for the DIABLO models is complex and, likely, the current study is not sufficient powered. These considerations are important to consider when interpreting the results of the current work. The exploratory nature of this study should be utilized to generate hypotheses for future study development. After initiation of future studies, validation studies are needed to confirm these findings. The current study is therefore only the preliminary evidence, and this should be considered when interpreting the data.

Conclusion

The current study is the first human study to utilize the DIABLO model to explain clinical pain intensity 5-years after TKA surgery and to demonstrate that measures of pain sensitivity, psychological factors, microRNAs, and inflammation are interconnected and can form pain mechanistic networks associated with clinical pain intensity in the investigated cohort. In the current work, four different models are presented ranging from a complex model including 40 parameters to less complex model including 5 parameters. The complex model explained 81% while the less complex model explained 51% of the variability of clinical pain intensity. The current work should serve as an example of how complex data can be analyzed using supervised machine-learning techniques, but the results should be validated in external cohorts in the future.

Acknowledgment

The Center for Neuroplasticity and Pain (CNAP) is supported by the Danish National Research Foundation (DNRF121). The Center for Mathematical Modeling of Knee Osteoarthritis (MathKOA) is funded by the Novo Nordisk Foundation (NNF21OC0065373). Gigtforeningen (The Danish Rheumatism Association) is acknowledged for the support. The Danish Rheumatism Association (A7645). The authors would like to acknowledge Prof. Ole Simonsen as the initiator of the study. M.C. Gerra is supported by a fixed-term research assistant contract by the COMP-HUB Initiative, funded by the 'Departments of Excellence' program of the Italian Ministry for Education, University and Research (MIUR, 2018-2022). This research was granted by the University of Parma through the action Bando di Ateneo 2023 per la ricerca.

Data relative to this work will be available upon reasonable request to the corresponding author.

Author Contribution

LAN, AK, SEØ, and KKP designed the study, SEØ and AK collected the data, RG, MCG, CD and CC conducted the experimental inflammatory analysis, RG conducted the experimental miRNAs analysis and conducted the statistical analyses, RG, LAN and KKP contributed to the interpretation of data and RG drafted the manuscript. All authors critically reviewed the manuscript and approved the final version of the manuscript before submission.

Reference list

[1] Ahmad L, Demartini C, Corrado M, Vaghi G, Piella EM, Allena M, Zanaboni AM, Greco R, Tassorelli C, De Icco R. Expression of Selected microRNAs in Migraine: A New Class of Possible Biomarkers of Disease? Processes 2021, Vol 9, Page 2199 2021;9:2199. doi:10.3390/PR9122199.

[2] Arden NK, Perry TA, Bannuru RR, Bruyère O, Cooper C, Haugen IK, Hochberg MC, McAlindon TE, Mobasheri A, Reginster JY. Non-surgical management of knee osteoarthritis: comparison of ESCEO and OARSI 2019 guidelines. Nature Reviews Rheumatology 2020 17:1 2020;17:59–66. doi:10.1038/s41584-020-00523-9.

[3] Arendt-Nielsen L, Egsgaard LL, Petersen KK, Eskehave TN, Graven- Nielsen T, Hoeck HC, Simonsen O. A mechanism-based pain sensitivity index to characterize knee osteoarthritis patients with different disease stages and pain levels. European Journal of Pain 2015;19:1406–1417. doi:10.1002/EJP.651.

[4] Arendt-Nielsen L, Eskehave TN, Egsgaard LL, Petersen KK, Graven-Nielsen T, Hoeck HC, Simonsen O, Siebuhr AS, Karsdal M, Bay-Jensen AC. Association Between Experimental Pain Biomarkers and Serologic Markers in Patients With Different Degrees of Painful Knee Osteoarthritis. Arthritis & Rheumatology 2014;66:3317–3326. doi:10.1002/ART.38856.

[5] Arendt-Nielsen L, Morlion B, Perrot S, Dahan A, Dickenson A, Kress HG, Wells C, Bouhassira D, Mohr Drewes A. Assessment and manifestation of central sensitisation across different chronic pain conditions. European Journal of Pain 2018;22:216–241. doi:10.1002/EJP.1140.

[6] Arendt-Nielsen L, Nie H, Laursen MB, Laursen BS, Madeleine P, Simonsen OH, Graven-Nielsen T. Sensitization in patients with painful knee osteoarthritis. Pain 2010;149:573–581. doi:10.1016/J.PAIN.2010.04.003.

[7] Argelaguet R, Velten B, Arnol D, Dietrich S, Zenz T, Marioni JC, Buettner F, Huber W, Stegle O. Multi-Omics Factor Analysis—a framework for unsupervised integration of multi-omics data sets. Mol Syst Biol 2018;14:8124. doi:10.15252/MSB.20178124/SUPPL_FILE/MSB178124-SUP-0002-EVFIGS.PDF.

[8] Bäckryd E, Themistocleous A, Larsson A, Gordh T, Rice ASC, Tesfaye S, Bennett DL, Gerdle B. Hepatocyte growth factor, colony-stimulating factor 1, CD40, and 11 other inflammation-related proteins are associated with pain in diabetic neuropathy: exploration and replication serum data from the Pain in Neuropathy Study. Pain 2022;163:897. doi:10.1097/J.PAIN.0000000000002451.

[9] Baker K, Grainger A, Niu J, Clancy M, Guermazi A, Crema M, Hughes L, Buckwalter J, Wooley A, Nevitt M, Felson DT. Relation of synovitis to knee pain using contrast-enhanced MRIs. Ann Rheum Dis 2010;69:1779–1783. doi:10.1136/ARD.2009.121426.

[10] Bali KK, Gandla J, Rangel DR, Castaldi L, Mouritzen P, Agarwal N, Schmelz M, Heppenstall P, Kuner R. A genome-wide screen reveals microRNAs in peripheral sensory neurons driving painful diabetic neuropathy. Pain 2021;162:1334–1351.

[11] Beswick AD, Wylde V, Gooberman-Hill R, Blom A, Dieppe P. What proportion of patients report long-term pain after total hip or knee replacement for osteoarthritis? A systematic review of prospective studies in unselected patients. BMJ Open 2012;2:e000435. doi:10.1136/BMJOPEN-2011-000435.

[12] Boonstra AM, Stewart RE, Albère AJ, René RF, Swaan JL, Schreurs KMG, Schiphorst Preuper HR. Cut-offpoints for mild, moderate, and severe pain on the numeric rating scale for pain in patients with chronic musculoskeletal pain: Variability and influence of sex and catastrophizing. Front Psychol 2016;7:212592. doi:10.3389/FPSYG.2016.01466/BIBTEX.

[13] Campbell CM, Buenaver LF, Finan P, Bounds SC, Redding M, McCauley L, Robinson M, Edwards RR, Smith MT. Sleep, Pain Catastrophizing, and Central Sensitization in Knee Osteoarthritis Patients With and Without Insomnia. Arthritis Care Res (Hoboken) 2015;67:1387–1396. doi:10.1002/ACR.22609.

[14] Christensen KS, O'Sullivan K, Palsson TS. Conditioned Pain Modulation Efficiency Is Associated With Pain Catastrophizing in Patients With Chronic Low Back Pain. Clin J Pain 2020;36:825–832.

[15] Churov A V., Oleinik EK, Knip M. MicroRNAs in rheumatoid arthritis: Altered expression and diagnostic potential. Autoimmun Rev 2015;14:1029–1037.

[16] Doehring A, Geisslinger G, Lötsch J. Epigenetics in pain and analgesia: an imminent research field. Eur J Pain 2011;15:11–6.

[17] Dyck PJ, Zimmerman I, Gillen DA, Johnson D, Karnes JL, O'Brien PC. Cool, warm, and heat-pain detection thresholds: testing methods and inferences about anatomic distribution of receptors. Neurology 1993;43:1500–8.

[18] Edwards RR, Cahalan C, Mensing G, Smith M, Haythornthwaite J a. Pain, catastrophizing, and depression in the rheumatic diseases. Nat Rev Rheumatol 2011;7:216–224.

[19] Edwards RR, Calahan C, Mensing G, Smith M, Haythornthwaite JA. Pain, catastrophizing, and depression in the rheumatic diseases. Nature Reviews Rheumatology 2011 7:4 2011;7:216–224. doi:10.1038/nrrheum.2011.2.

[20] Eitner A, Pester J, Vogel F, Marintschev I, Lehmann T, Hofmann GO, Schaible H-G. Pain sensation in human osteoarthritic knee joints is strongly enhanced by diabetes mellitus. Pain 2017;158:1743–1753.

[21] Friedman Y, Balaga O, Linial M. Working together: combinatorial regulation by microRNAs. Adv Exp Med Biol 2013;774:317–337. doi:10.1007/978-94-007-5590-1_16.

[22] Gallelli L, Cione E, Peltrone F, Siviglia S, Verano A, Chirchiglia D, Zampogna S, Guidetti V, Sammartino L, Montana A, Caroleo MC, De Sarro G, Di Mizio G. Hsa-miR-34a-5p and hsa-miR-375 as Biomarkers for Monitoring the Effects of Drug Treatment for Migraine Pain in Children and Adolescents: A Pilot Study. Journal of Clinical Medicine 2019, Vol 8, Page 928 2019;8:928. doi:10.3390/JCM8070928.

[23] Gandhi R, Santone D, Takahashi M, Dessouki O, Mahomed NN. Inflammatory predictors of ongoing pain 2 years following knee replacement surgery. Knee 2013;20:316–318.

[24] Géranton SM, Tochiki KK. Regulation of gene expression and pain states by epigenetic mechanisms. Prog Mol Biol Transl Sci 2015;131:147–83.

[25] Giordano R, Gerra MC, Okutani H, Lo Vecchio S, Stensballe A, Petersen KK, Arendt-Nielsen L. The temporal expression of circulating microRNAs after acute experimental pain in humans. European Journal of Pain 2022.

[26] Giordano R, Ghafouri B, Arendt-Nielsen L, Petersen KK. Inflammatory biomarkers in patients with painful knee osteoarthritis: exploring the potential link to chronic postoperative pain after total knee arthroplasty-a secondary analysis. Pain 2024;165:337–346.

[27] Giordano R, Ghafouri B, Arendt-Nielsen L, Petersen KK-S. Inflammatory biomarkers in patients with painful knee osteoarthritis: exploring the potential link to chronic postoperative pain after total knee arthroplasty—a secondary analysis. Pain 2023. doi:10.1097/j.pain.0000000000003042.

[28] Giordano R, Kjær-Staal Petersen K, Arendt-Nielsen L. The link between epigenetics, pain sensitivity and chronic pain. Scand J Pain 2022;22:664–666.

[29] Giordano R;, Petersen K, Kjaer ;, Andersen H, Holm ;, Lichota J;, Valeriani M;, Simonsen O;, Arendt-Nielsen L, Giordano R, Petersen KK, Andersen HH, Valeriani J, Simonsen M, Arendt-Nielsen &, Giordano R, Petersen KK, Holm Andersen H, Lichota J, Valeriani M, Simonsen O. Preoperative serum circulating microRNAs as potential biomarkers for chronic postoperative pain after total knee replacement. Mol Pain 2020;16. doi:10.1177/1744806920962925.

[30] Giordano R, Petersen KK, Andersen HH, Lichota J, Valeriani M, Simonsen O, Arendt-Nielsen L. Preoperative serum circulating microRNAs as potential biomarkers for chronic postoperative pain after total knee replacement. Mol Pain 2020;16:1744806920962925.

[31] Giordano R, Petersen KK, Andersen HH, Lichota J, Valeriani M, Simonsen O, Arendt-Nielsen L. Preoperative serum circulating microRNAs as potential biomarkers for chronic postoperative pain after total knee replacement. Mol Pain 2020;16:174480692096292. doi:10.1177/1744806920962925.

[32] Giordano R, Petersen KKS, Arendt-Nielsen L. The link between epigenetics, pain sensitivity and chronic pain. Scand J Pain 2022;22:664–666. doi:10.1515/SJPAIN-2022-0086/MACHINEREADABLECITATION/RIS.

[33] Harris K, Dawson J, Doll H, Field RE, Murray DW, Fitzpatrick R, Jenkinson C, Price AJ, Beard DJ. Can pain and function be distinguished in the Oxford Knee Score in a meaningful way? An exploratory and confirmatory factor analysis. Quality of Life Research 2013;22:2561–2568. doi:10.1007/S11136-013-0393-X/TABLES/4.

[34] Hertel E, Arendt-Nielsen L, Olesen A, Andersen M, Petersen KK-S. Quantitative Sensory Testing, Psychological Factors, and Quality of Life as Predictors of Current and Future Pain in Patients with Knee Osteoarthritis. Pain 2024.

[35] Hertel E, McPhee ME, Petersen KK. Investigation of pain sensitivity following 3 nights of disrupted sleep in healthy individuals. European Journal of Pain 2023;27:710–722. doi:10.1002/EJP.2101.

[36] Hill CL, Hunter DJ, Niu J, Clancy M, Guermazi A, Genant H, Gale D, Grainger A, Conaghan P, Felson DT. Synovitis detected on magnetic resonance imaging and its relation to pain and cartilage loss in knee osteoarthritis. Ann Rheum Dis 2007;66:1599. doi:10.1136/ARD.2006.067470.

[37] Hollins SL, Cairns MJ. MicroRNA: Small RNA mediators of the brains genomic response to environmental stress. Prog Neurobiol 2016;143:61–81.

[38] Keogh E. Sex and gender differences in pain: past, present, and future. Pain 2022;163:S108–S116. doi:10.1097/J.PAIN.0000000000002738.

[39] Khalatbari-Soltani S, Blyth FM. Socioeconomic position and pain: A topical review. Pain 2022;163:1855–1861. doi:10.1097/J.PAIN.0000000000002634.

[40] Kovanur Sampath K, Belcher S, Hales J, Thomson OP, Farrell G, Gisselman AS, Katare R, Tumilty S. The role of micro-RNAs in neuropathic pain—a scoping review. Pain Rep 2023;8:E1108. doi:10.1097/PR9.0000000000001108.

[41] Kurien T, Kerslake RW, Graven-Nielsen T, Arendt-Nielsen L, Auer DP, Edwards K, Scammell BE, Petersen KKS. Chronic postoperative pain after total knee arthroplasty: The potential contributions of synovitis, pain sensitization and pain catastrophizing—An explorative study. European Journal of Pain (United Kingdom) 2022;26:1979–1989. doi:10.1002/ejp.2018.

[42] Lê Cao KA, González I, Déjean S. integrOmics: an R package to unravel relationships between two omics datasets. Bioinformatics 2009;25:2855–2856. doi:10.1093/BIOINFORMATICS/BTP515.

[43] Li W, Zhang S, Liu CC, Zhou XJ. Identifying multi-layer gene regulatory modules from multi-dimensional genomic data. Bioinformatics 2012;28:2458–2466. doi:10.1093/BIOINFORMATICS/BTS476.

[44] Lively S, Milliot M, Potla P, Espin-Garcia O, Layeghifard M, Sundararajan K, Endisha H, Nakamura A, Perruccio A V., Veillette C, Kapoor M, Rampersaud YR. Association of presurgical circulating MicroRNAs with 1-year postsurgical pain reduction in spine facet osteoarthritis patients with lumbar spinal stenosis. Osteoarthr Cartil Open 2022;4:100283.

[45] López-Muñoz E, Mejía-Terrazas GE. Epigenetics and Postsurgical Pain: A Scoping Review. Pain Medicine 2022;23:246–262. doi:10.1093/PM/PNAB234.

[46] Lyng KD, Andersen JD, Jensen SL, Olesen JL, Arendt-Nielsen L, Madsen NK, Petersen KK. The influence of exercise on clinical pain and pain mechanisms in patients with subacromial pain syndrome. Eur J Pain 2022;26. doi:10.1002/EJP.2010.

[47] Mauceri D. Role of Epigenetic Mechanisms in Chronic Pain. Cells 2022;11. doi:10.3390/cells11162613.

[48] Murray DW, Fitzpatrick R, Rogers K, Pandit H, Beard DJ, Carr AJ, Dawson J. The use of the Oxford hip and knee scores. Journal of Bone and Joint Surgery - Series B 2007;89:1010–1014.

[49] Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, Asahara H. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. Arthritis Rheum 2008;58:1284–1292.

[50] Petersen CL, Kjærsgaard JB, Kjærgaard N, Jensen MU, Laursen MB. Thresholds for Oxford Knee Score after total knee replacement surgery: a novel approach to post-operative evaluation. J Orthop Surg Res 2017;12. doi:10.1186/S13018-017-0592-1.

[51] Petersen KK, Arendt-Nielsen L, Simonsen O, Wilder-Smith O, Laursen MB. Presurgical assessment of temporal summation of pain predicts the development of chronic postoperative pain 12 months after total knee replacement. Pain 2015;156:55–61. doi:10.1016/J.PAIN.0000000000000022.

[52] Petersen KK, Vaegter HB, Stubhaug A, Wolff A, Scammell BE, Arendt-Nielsen L, Larsen DB. The predictive value of quantitative sensory testing: a systematic review on chronic postoperative pain and the analgesic effect of pharmacological therapies in patients with chronic pain. Pain 2021;162:31–44. doi:10.1097/J.PAIN.0000000000002019.

[53] Petersen KK-S. Predicting pain after standard pain therapy for knee osteoarthritis - the first steps towards personalized mechanistic-based pain medicine in osteoarthritis. Scand J Pain 2023;23:40–48.

[54] Petersen KKS. Predicting pain after standard pain therapy for knee osteoarthritis - the first steps towards personalized mechanistic-based pain medicine in osteoarthritis. Scand J Pain 2022;23:40–48. doi:10.1515/SJPAIN-2022-0082.

[55] Pogatzki-Zahn EM, Segelcke D. Searching for the rainbow: biomarkers relevant for chronic postsurgical pain. Pain 2023. doi:10.1097/J.PAIN.0000000000003043.

[56] Reinhold AK, Salvador E, Förster CY, Birklein F, Rittner HL. Microvascular Barrier Protection by microRNA-183 via FoxO1 Repression: A Pathway Disturbed in Neuropathy and Complex Regional Pain Syndrome. J Pain 2022;23:967–980.

[57] Rohart F, Gautier B, Singh A, Lê Cao KA. mixOmics: An R package for 'omics feature selection and multiple data integration. PLoS Comput Biol 2017;13. doi:10.1371/JOURNAL.PCBI.1005752.

[58] Rolke R, Magerl W, Campbell KA, Schalber C, Caspari S, Birklein F, Treede RD. Quantitative sensory testing: A comprehensive protocol for clinical trials. European Journal of Pain 2006;10:77–88.

[59] Schaible HG. Nociceptive neurons detect cytokines in arthritis. Arthritis Res Ther 2014;16:1–9. doi:10.1186/s13075-014-0470-8.

[60] Schaible H-G. Spinal mechanisms contributing to joint pain. Novartis Found Symp 2004;260:4–22; discussion 22-7, 100–4, 277–9.

[61] Schaible HG, Grubb BD. Afferent and spinal mechanisms of joint pain. Pain 1993;55:5–54.

[62] Schaible HG, Grubb BD. Afferent and spinal mechanisms of joint pain. Pain 1993;55:5–54. doi:10.1016/0304-3959(93)90183-P.

[63] Schaible H-GG. Nociceptive neurons detect cytokines in arthritis. Arthritis Res Ther 2014;16:470–478.

[64] Siebuhr AS, Petersen KK, Arendt-Nielsen L, Egsgaard LL, Eskehave T, Christiansen C, Simonsen O, Hoeck HC, Karsdal MA, Bay-Jensen AC. Identification and characterisation of osteoarthritis patients with inflammation derived tissue turnover. Osteoarthritis Cartilage 2014;22:44–50.

[65] Singh A, Shannon CP, Gautier B, Rohart F, Vacher M, Tebbutt SJ, Cao KAL. DIABLO: an integrative approach for identifying key molecular drivers from multi-omics assays. Bioinformatics 2019;35:3055. doi:10.1093/BIOINFORMATICS/BTY1054.

[66] Skrejborg P, Petersen KK, Kold S, Kappel A, Pedersen C, Østgaard SE, Simonsen O, Arendt-Nielsen L. Patients with High Chronic Postoperative Knee Pain 5 Years after Total Knee Replacement Demonstrate Low-grad Inflammation, Impairment of Function, and High Levels of Pain Catastrophizing. Clinical Journal of Pain 2021;37:161–167. doi:10.1097/AJP.0000000000000907.

[67] Sommer C, Leinders M, Üçeyler N. Inflammation in the pathophysiology of neuropathic pain. Pain 2018;159:595–602. doi:10.1097/j.pain.0000000000001122.

[68] Staffe AT, Bech MW, Clemmensen SLK, Nielsen HT, Larsen DB, Petersen KK. Total sleep deprivation increases pain sensitivity, impairs conditioned pain modulation and facilitates temporal summation of pain in healthy participants. PLoS One 2019;14:e0225849. doi:10.1371/JOURNAL.PONE.0225849.

[69] Sullivan MJL, Bishop SR, Pivik J. The Pain Catastrophizing Scale: Development and Validation. Psychol Assess 1995;7:524–532.

[70] Tavares-Ferreira D, Lawless N, Bird E V., Atkins S, Collier D, Sher E, Malki K, Lambert DW, Boissonade FM. Correlation of miRNA expression with intensity of neuropathic pain in man. Mol Pain 2019;15. doi:10.1177/1744806919860323/ASSET/IMAGES/LARGE/10.1177_1744806919860323-FIG6.JPEG.

[71] Tenenhaus A, Philippe C, Guillemot V, Le Cao KA, Grill J, Frouin V. Variable selection for generalized canonical correlation analysis. Biostatistics 2014;15:569–583. doi:10.1093/BIOSTATISTICS/KXU001.

[72] Wan L, Su Z, Li F, Gao P, Zhang X. MiR-122-5p suppresses neuropathic pain development by targeting PDK4. Neurochem Res 2021;46:957–963. doi:10.1007/S11064-020-03213-W/FIGURES/4.

[73] Wang H, Sun Y, Wu Y, Shen YJ, Xia J, Xu YH, Yue L, Sun L, Li XJ, Ma Y, Xiong YC, Li D. MiR-30d Participates in Vincristine-Induced Neuropathic Pain by Down-Regulating GAD67. Neurochem Res 2022;47:481–492. doi:10.1007/S11064-021-03462-3/FIGURES/4.

[74] Wang J, Chen J, Sen S. MicroRNA as Biomarkers and Diagnostics. J Cell Physiol 2016;231:25–30.

[75] Wen Q, Wang Y, Pan Q, Tian R, Zhang D, Qin G, Zhou J, Chen L. MicroRNA-155-5p promotes neuroinflammation and central sensitization via inhibiting SIRT1 in a nitroglycerin-induced chronic migraine mouse model. J Neuroinflammation 2021;18:287. doi:10.1186/s12974-021-02342-5.

[76] Wilder-Smith OH. A paradigm-shift in pain medicine. Aalborg: Center for Sensory-Motor Interaction (SMI), Department of Health Science and Technology, Aalborg University, 2013.

[77] Zajacova A, Rogers RG, Grodsky E, Grol-Prokopczyk H. The Relationship between Education and Pain among Adults Aged 30-49 in the United States HHS Public Access. J Pain 2020;21:1270–1280.

[78] Zhang G, Zhou Y, Su M, Yang X, Zeng B. Inhibition of microRNA-27b-3p relieves osteoarthritis pain via regulation of KDM4B-dependent DLX5. BioFactors 2020;46:788–802. doi:10.1002/BIOF.1670.

Tables

Table 1. Demographics of evaluated patients. The table displays the data average and standard deviation for age, BMI, and pain intensity (VAS0-10) of all patients included in the studied cohort.

Age (at follow-up)	70.78 ± 7.64
Gender	43 females; 32 males
Body Mass Index (BMI)	30.94 ± 5.53
Pain intensity ($VAS_{0\text{-}10}$)	4.45 ± 2.50

Table 2. Comparative analysis of DIABLO approaches for evaluation of the efficiency of variable reduction and model performance. Comparison between two DIABLO methods with and without backward elimination, highlighting the number of variables and the R^2 values that represent the model's specific predictive value.

	3 groups		**2 groups**	
Approach	DIABLO	DIABLO+ Backward elimination	DIABLO	DIABLO+ Backward elimination
Variables number	40	12	21	5
R^2	81%	69%	63%	51%

Table 3. Linear regression models of 40 variables selected by DIABLO analysis for the explanation of clinical pain intensity in patients with knee osteoarthritis 5 years after total knee replacement. Model 1 consists of all the parameters and R^2 indicates the combined predictive value. Model 2 applies backward elimination reporting independent factors to explain clinical pain intensity and R^2 indicates the combined predictive value.

Model	**Variable**	**Standardized Coefficients**	**p-value**	**R2**
1				0.81
	PDT	0.009	0.940	
	CPM	0.191	0.123	
	TS	-0.039	0.741	
	PCS	0.283	0.081	

	OKS-pain	-0.328	0.018	
	hsa-miR-1304-3p	0.062	0.658	
	hsa-miR-431-5p	0.042	0.766	
	hsa-miR-708-3p	0.211	0.138	
	hsa-miR-3164	0.131	0.428	
	hsa-miR-433-3p	0.153	0.278	
	hsa-miR-143-3p	-0.219	0.157	
	hsa-miR-4741	0.114	0.316	
	hsa-miR-3678-3p	-0.231	0.118	
	hsa-miR-30d-3p	0.113	0.276	
	hsa-miR-27b-3p	0.418	0.043	
	hsa-miR-217-5p	0.473	0.008	
	hsa-miR-4479	-0.160	0.254	
	hsa-miR-654-3p	0.150	0.335	
	hsa-miR-5189-5p	-0.045	0.695	
	hsa-miR-487b-3p	-0.186	0.165	
	hsa-miR-193b-5p	-0.370	0.326	
	hsa-miR-34a-5p	0.002	0.993	
	hsa-miR-122-5p	0.010	0.975	
	hsa-miR-324-5p	-0.125	0.439	
	hsa-miR-5004-5p	-0.044	0.760	
	EGF	-0.195	0.532	
	GCSF	-0.164	0.517	
	IFNa	0.386	0.357	
	IL1a	-0.153	0.641	
	IL1ra	0.030	0.827	
	IL6	-0.129	0.629	
	IL8	0.195	0.148	
	IL13	-0.699	0.046	
	IL15	-0.431	0.010	
	MIP1b	0.059	0.833	
	PDGFAA	-0.362	0.411	
	PDGFAB/BB	0.873	0.014	
	RANTES	0.135	0.634	
	TGFa	0.328	0.104	
	VEGF	0.329	0.030	
2				**0.69**
	CPM	0.135	0.096	
	PCS	0.391	<0.001	
	OKS-pain	-0.379	<0.001	
	hsa-miR-27b-3p	0.319	0.009	

hsa-miR-217-5p	0.245	0.006
hsa-miR-4479	-0.209	0.019
hsa-miR-193b-5p	-0.306	0.007
IL13	-0.509	0.002
IL15	-0.369	0.004
PDGFAB/BB	0.476	0.004
TGFa	0.465	<0.001
VEGF	0.301	0.001

Table 4. Linear regression models of 21 variables selected by DIABLO analysis for the explanation of clinical pain intensity in patients with knee osteoarthritis 5 years after total knee replacement based on 2 groups separation. Model 1 consists of all the parameters and R^2 indicates the combined predictive value. Model 2 applies backward elimination reporting independent factors to explain clinical pain intensity and R^2 indicates the combined predictive value.

Model	Variable	Standardized Coefficients	p-value	R2
1				**0.63**
	PDT	-0.189	0.007	
	CPM	0.105	0.094	
	TS	-0.115	0.290	
	PCS	0.352	0.081	
	OKS-func	-0.216	0.018	
	OKS-pain	-0.158	0.658	
	hsa-miR-6736-5p	0.013	0.897	
	hsa-miR-1343-5p	-0.131	0.161	
	hsa-miR-654-3p	-0.075	0.463	
	hsa-miR-217-5p	0.247	0.029	
	hsa-miR-3678-3p	0.033	0.756	
	hsa-miR-30d-5p	-0.157	0.192	

	hsa-miR-3679-5p	0.148	0.146	
	hsa-miR-1304-3p	0.153	0.163	
	hsa-miR-27b-3p	0.069	0.568	
	hsa-miR-3164	0.169	0.192	
	GCSF	-0.420	0.037	
	IL1ra	-0.205	0.075	
	IL6	0.147	0.322	
	MIP1b	0.116	0.451	
	RANTES	0.212	0.201	
2				**0.51**
	PCS	0.343	0.002	
	OKS-pain	-0.396	<0.001	
	hsa-miR-217-5p	0.211	0.029	
	GCSF	-0.260	0.073	
	RANTES	0.347	0.010	

FIGURE LEGENDS

FIGURE 1. Circos plot from multiblock sPLS-DA for component 1 in 3 groups separation. The plots represent the correlations greater than 0.5 between variables selected for all parameters. The internal connecting lines show the positive (red) and negative (blue) inter-correlations. The outer lines show the expression levels of each variable in each sample group, none-to-mild (dark green), moderate (blue), and severe (red) clinical pain intensity.

FIGURE 2. Circos plot from multiblock sPLS-DA for component 1 in 2 groups separation. The plots represent the correlations greater than 0.5 between variables selected for all parameters. The internal connecting lines show the positive (red) and negative (blue) inter-correlations. The outer lines show the expression levels of each variable in each sample group, none-to-mild (dark green), moderate-to-severe (red) clinical pain intensity.

Article summary

A multifactorial combination of variables including clinical, functional, cognitive, and biological factors can explain pain intensity in patients with osteoarthritis 5-years after surgery.

Figure 1

Click here to access/download;Figure;newcircos3groups.tiff

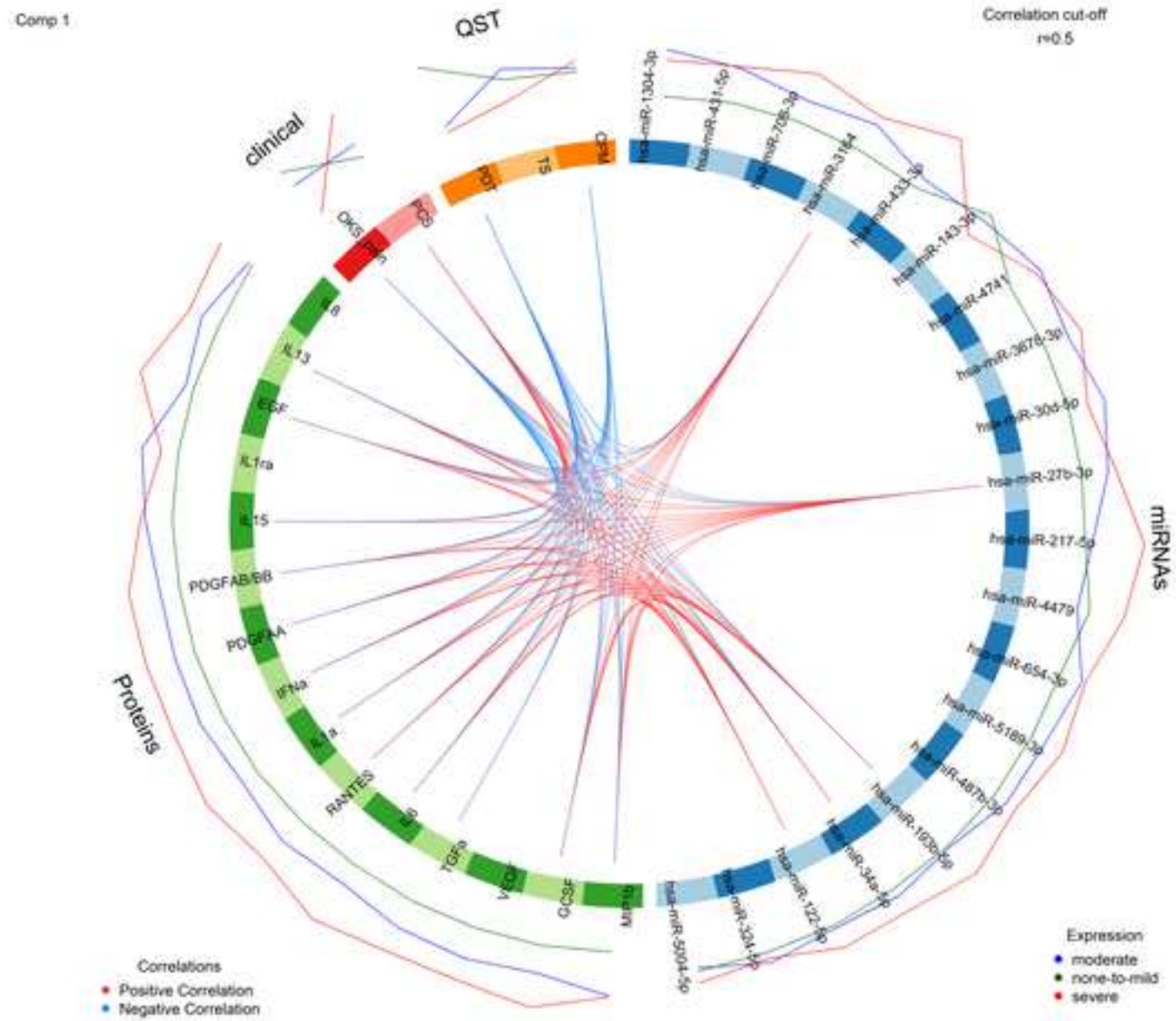
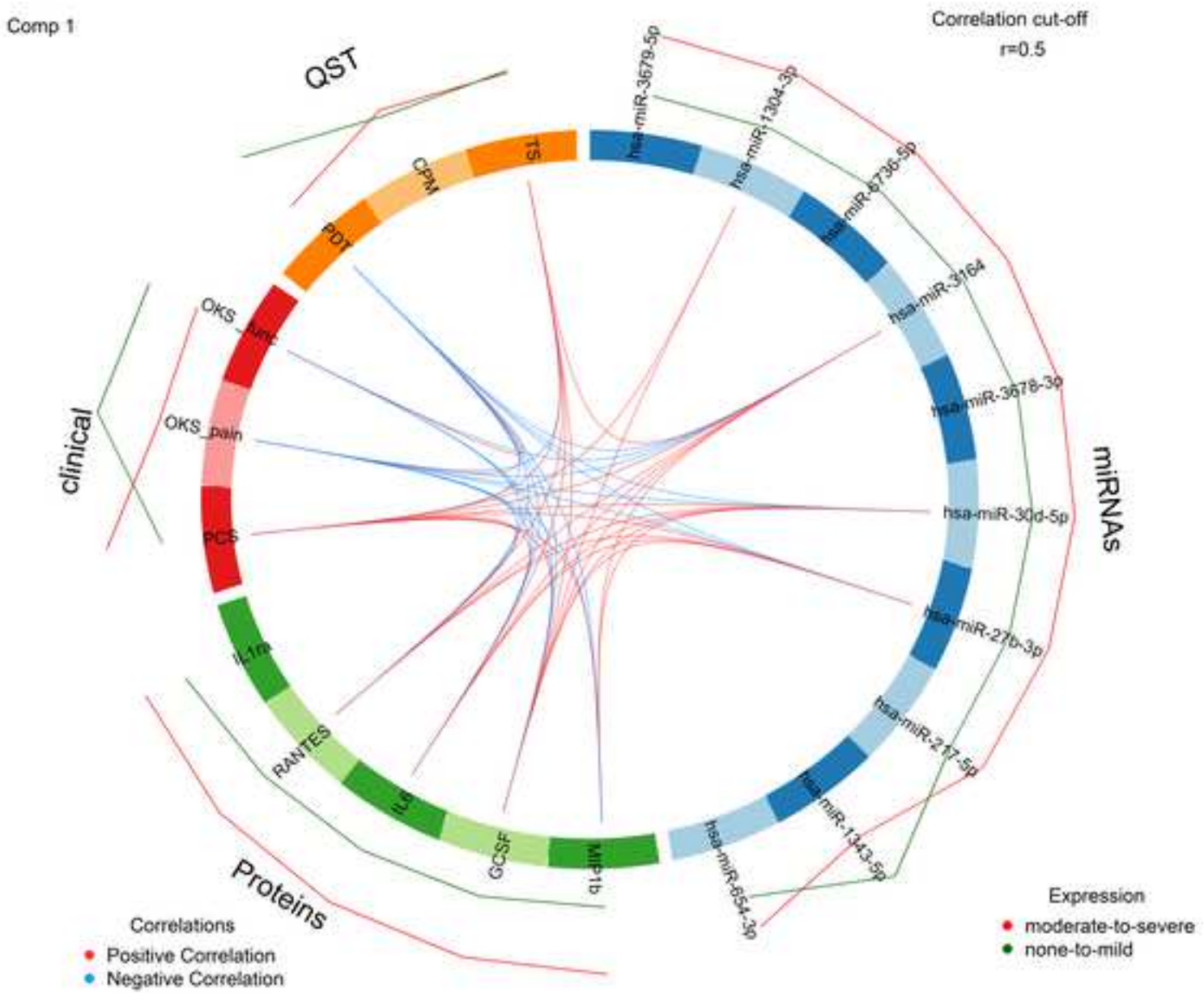


Figure 2

Click here to access/download;Figure;newcircos2groups.tiff



STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Abstract and page 1
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	1
Objectives	3	State specific objectives, including any prespecified hypotheses	2
Methods			
Study design	4	Present key elements of study design early in the paper	2
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	2
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up (*b*) For matched studies, give matching criteria and number of exposed and unexposed	2
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	2-5
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	2-5
Bias	9	Describe any efforts to address potential sources of bias	5-6
Study size	10	Explain how the study size was arrived at	12
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5-6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding (*b*) Describe any methods used to examine subgroups and interactions (*c*) Explain how missing data were addressed (*d*) If applicable, explain how loss to follow-up was addressed (*e*) Describe any sensitivity analyses	5-6
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	7
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)	7
Outcome data	15*	Report numbers of outcome events or summary measures over time	7-9

Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (*b*) Report category boundaries when continuous variables were categorized (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	7-9
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	NA
Discussion			
Key results	18	Summarise key results with reference to study objectives	9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	12
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9-12
Generalisability	21	Discuss the generalisability (external validity) of the study results	9-12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	13

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at http://www.strobe-statement.org.

Supplementary figure 1

Supplementary figure 2-9