Responders vs. non-responders to mesenchymal stromal cells in knee osteoarthritis patients: mechanistic correlates of donor cell attributes and patient features

Running title: Mechanistic correlates to MSCs in knee osteoarthritis

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ABSTRACT

Background: Despite recent regulatory approvals for mesenchymal stromal cells (MSCs), including two products for knee and degenerative arthritis globally, MSCs have shown mixed efficacy in advanced clinical trials of knee osteoarthritis (KOA). Analysis of contributing factors to the underlying heterogeneity in patient responsiveness is needed to comprehend these mixed results and to advance clinical translation.

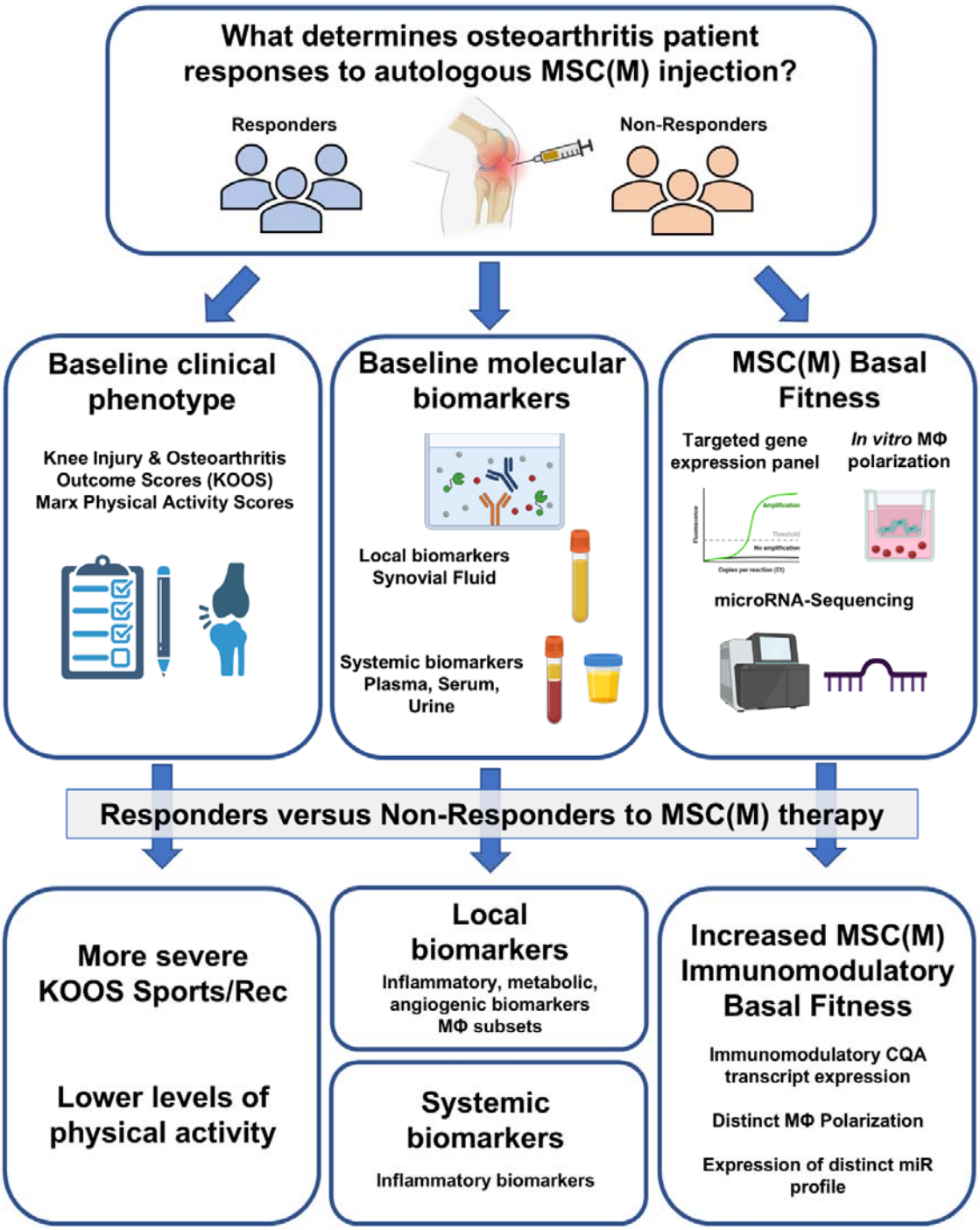
Methods: We provide the basis for a novel algorithm that correlates with responder (defined by stringent international consensus criteria) vs. non-responder classifications using i) the immunomodulatory baseline features of bone marrow-derived MSCs (MSC(M)) batches, and ii) differential KOA baseline clinical features, local and systemic biomarkers. We use our previous clinical trial with twelve batches of MSC(M) as a test dataset and demonstrate correlations with new longitudinal medium-and long-term clinical data.

Results: We report that KOA clinical responsiveness to MSC treatment is dictated by a combination of the immunomodulatory fitness of the donor MSC(M) batches, clinical phenotype, baseline immune cell profile, and biomarker profile. Specifically, MSC(M) in KOA clinical responders have reduced expression of angiogenic genes, *THBS1*, *CXCL8*, and *ANGPT1* and higher expression of growth factor *CCN2* and distinct microRNA signatures, as well as higher *in vitro* ability to modulate monocytes that significantly distinguish them from MSC(M) from non-responders. Clinical responders had greater OA baseline severity, reduced physical activity; lower local baseline levels of CCL2, VEGFA, HGF; higher local baseline of resistin; and putatively lower frequencies of joint-specific intermediate monocytes/macrophages and activated CD4 T cells. Leave-out-one sensitivity analysis confirmed these findings mitigating small sample size concerns. Importantly, predicted responder status at 12 and 24 months was maintained out to 8-10 years in 5/7 responders and 3/3 non-responders, confirming the utility of our approach.

Conclusions: We provide a systematic, quantitative, evidence-based method for analyzing heterogeneity in KOA patient responsiveness to MSC treatments, dually incorporating batch/donor and patient factors. Our combinatorial analytical method provides a powerful framework for parsing heterogeneity in KOA patient responsiveness to MSC therapies and defining critical quality attributes that can reliably identify potent batches and/or donors of MSCs.

Significance statement: Using our data-rich clinical trial with 12-, 24- months and 8-10-year longitudinal follow-up data, we assess donor/batch cell attributes and patient factors that drive clinical efficacy. Bone Marrow MSC(M) from clinical responders exhibited lower angiogenic gene expression, greater ability to modulate monocytes and differential microRNA expression. Responsive KOA patients had more severe KOA, lower physical activity levels, and lower angiogenic proteins in their joints at baseline. Our quantitative analytical framework can be used prospectively in designing future clinical trials and retrospectively in analyzing MSC effectiveness in KOA patients.

Graphical Abstract



INTRODUCTION

Knee osteoarthritis (KOA) affects 16% of adults over 40 years of age worldwide [1], with no available disease-modifying drugs. Pathophysiological mechanisms of KOA are heterogeneous and include abnormal mechanical loading, metabolic, and inflammatory factors that fluctuate temporally [2]. Culture-expanded mesenchymal stromal cells (MSCs) are being investigated for their multimodal mechanisms of action, including chondroprotective/cartilage reparative, anti-fibrotic, and immunomodulatory functions [3,4]However, there has been mixed clinical efficacy reported including Mautner *et al*. who showed umbilical cord-derived MSCs were equivalent to corticosteroid injections in terms of improvements in pain or cartilage magnetic resonance imaging scores [5]. Other reports in smaller clinical trials [6–9], meta-analyses [10,11], and randomized controlled trials [12,13] however showed symptom and structural modification. These conflicting data raise questions: do MSCs work in KOA? Can we understand the differences between responder vs. non-responders to help deliver better MSC treatments to KOA patients?

Heterogeneous responsiveness to MSC treatments is due to an interplay of both basal potency fitness of donor MSC batches and basal patient disease status. Heterogeneity exists among MSC investigational products due to variations in donors [4,14] and manufacturing parameters [15,16]. The International Society for Cell & Gene Therapy (ISCT) has emphasized a need for well-defined critical quality attributes (CQAs) that are sensitive, quantitative, relevant to mechanism of action, and validated for specific disease indications [17,18] as we have also shown [19].

Using reported [8],and new follow-up clinical results from a phase I/IIa dose-escalation clinical trial, we conduct an in-depth combined analyses of donor MSC batch and baseline/follow up KOA patient characterization to provide a systematic algorithm to analyze heterogenous KOA patient responsiveness to MSC treatments. We use our previously reported gene panel that correlated to *in vitro* adipose tissue-derived MSC (MSC(AT)) functional activity [20], and show these discriminate MSC derived from marrow tissue, MSC(M), from responder vs. non-responder KOA patients. MicroRNAs, important mediators of MSC mechanisms of action [21] further discriminate MSC(M) from responders vs. non-responders.

In addition to MSC donor/batch heterogeneity, there is heterogeneity in KOA patients. KOA is now recognized not as a single disease but a constellation of clinical phenotypes (patient's clinical disease presentation [13]), including pain sensitization, metabolic syndrome, mechanical loading, and joint alignment [23,24]. KOA patients exhibit divergent molecular endotypes (molecular drivers of the disease) that may be characterized using local and/or systemic biomarkers [25]. The relationships between clinical phenotype and molecular endotype are complex and exhibit distinct or overlapping features, as well as temporal fluctuations [23,25]. This heterogeneity is a key contributor to the variable responses to MSC treatments. A systematic approach that incorporates baseline clinical phenotype and molecular disease endotype features that drive patient responsiveness are thus needed to improve MSC treatment outcomes.

MATERIALS AND METHODS

Clinical trial and patient data

KOA patients (Kellgren Lawrence (KL) grade 3-4) were recruited to this nonrandomized, open-label, dose-escalation, Health Canada-authorized phase I/IIa clinical trial as reported previously [8] (ClinicalTrials.gov Identifier: NCT02351011). Ethics approvals including for long-term follow up was granted by the UHN Research Ethics Board (REB 14–7909, REB 20-5066, REB 24-5463). Eligibility criteria and patient demographic information were previously provided [8]. Patients received a single intra-articular autologous MSC(M) injection at doses of 1 x 10^6, 10 x 10^6, and 50 x 10^6 cells (N=4 patients/dose group). Complete clinical follow-up was performed 24 months after MSC(M) injection for all patients. Additional follow-up, at 8-10 years was obtained for 10/12 patients; the remaining 2 patients were lost to follow-up; one patient (ID #3) could not be contacted and another (ID #10) underwent a total knee replacement in 2025 and declined to participate in the survey. Calculation of patient-reported outcome measures (PROMs), including the inverted KOOS and the Marx Activity Rating Scale (MARS) are described in **Suppl. File 1**.

Patient clinical responder status was based on the Outcome Measures in Rheumatology (OMERACT) and Osteoarthritis Research Society International (OARSI) Standing Committee for Clinical Trials Response Criteria Initiative (OMERACT-OARSI criteria). The OMERACT-OARSI criteria offers a rigorous responder classification system developed using data from large, powered placebo-controlled drug trials for hip and KOA [26]. These criteria categorize patients as responders according to the following: i) improvement in pain

or in function ≥50% and absolute change ≥ 20; OR, ii) improvement (≥20% and absolute change ≥ 10) in at least two of the following: a) pain, b) function, c) patient global assessment; the latter was not measured in our trial. Given the limited sample size and lack of patient global assessment metrics in our previous clinical trial, we categorized patient responder status using the twelve-month follow-up data based on changes in KOOS function (activities of daily living (ADL) subscale) and/or KOOS Pain only, applying OMERACT-OARSI criteria thresholds for function and pain scores of delta value ≥10 AND percentage change ≥20% [26]. The validated KOOS instrument measures responses to interventions in KOA with high test-retest reliability and improved sensitivity in pain and function scoring relative to the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) scoring system [26,27].

MSC(M) isolation and culture

Biobanked MSC(M) were retained as individual batches by consent of KOA patients and stored in liquid nitrogen. For microRNA-seq and gene expression analysis, MSC(M) were thawed in three batches at P3 or P4 and plated directly on 24-well plates as described below. For monocyte polarization experiments, MSC(M) were thawed in three batches at P4 or P5, expanded for one passage, and then plated for the assay as described below. MSC(M) culture was performed using the same expansion protocol and reagents used in the clinical trial [8] to minimize any batch processing parameters variations.

MSC(M) gene expression analysis

MSC(M) were seeded on 24-well plates at 30,000 cells/well and licensed with a cocktail of pro-inflammatory cytokines (IFNγ: 30 ng/mL; TNFα: 10 ng/mL; and IL-1β: 5 ng/mL), as previously described [20]. After 24 hours of culture, Trizol-chloroform extraction was performed to isolate RNA, and cDNA was generated using SuperScript™ IV VILO™ Master Mix (Invitrogen, Waltham, USA). FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) and custom primers (**Table S1**) were used for qPCR on a QuantStudio™ 5 system (ThermoFisher). Results were normalized (delta Ct; ΔCt) using reference genes (*GAPDH*, *GUSB*) and analyzed as negative ΔCt such that higher (more positive values) indicate a higher level of expression.

***In vitro* monocyte/macrophage polarization**

Indirect co-culture of unmatched human $CD14^+$ peripheral blood-derived monocytes (STEMCELL Technologies, Vancouver, Canada) with biobanked MSC(M) was performed as

previously described [20]. Co-culture was performed for 48 h and lipopolysaccharide (LPS) was spiked at a concentration of 2.5 ng/mL. After 4 h, monocytes were collected and RNA extraction, cDNA synthesis, and qPCR was performed as described above. Results were normalized (ΔΔCt) using reference genes (*GAPDH*, *B2M*, *TBP*) and presented as fold-change relative to monocytes cultured without MSC(M) (solo condition).

microRNA-sequencing on MSC(M) samples

Biobanked MSC(M) were thawed and plated on 24-well plates at 50,000 cells/well and cultured for 72 h in proliferation medium, supplemented with or without synovial fluid. Synovial fluid was collected from unrelated late-stage KOA patients (KL grade 3-4, REB #14–7483), pooled from eight patients, and stored at -80°C until use, as previously described [28]. For treated wells, medium was supplemented with 30% (v/v) synovial fluid. After 24 h, RNA extraction was performed using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) and stored at -30°C until analysis. Details on library preparation, microRNA-sequencing, and analysis are described in **Suppl. File 1**.

Biomarker measurements

Levels of curated biomarkers in serum (C1□2C collagen type I and II cleavage, C1-2C), plasma (hyaluronic acid, HA; cartilage oligomeric matrix protein, COMP), urine (C□telopeptide of type II collagen, CTX-II; collagen type II cleavage, C2C-HUSA), and synovial fluid (matrix metalloproteinases, MMP-3, MMP-9, MMP-13; tissue inhibitor of metalloproteinases 1, TIMP1; C□X□C chemokine motif ligands, CX3CL1, CXCL1; Chemokine C-C motif ligand 2 (CCL2); vascular endothelial growth factor A (VEGFA); IL12p40; interleukin(IL)-6; IL-8; hepatocyte growth factor (HGF); sCD14; sCD163; prostaglandin; adiponectin; adipsin; leptin; and resistin) collected at baseline prior to MSC(M) injection were measured in our previous study [8]. Updated analyses and experimental questions were applied to the dataset in the present work. Additional inflammatory biomarkers measured in biobanked patient serum and plasma collected at baseline were also evaluated by ELISA, with analytes specified in **Table S2**. Synovial fluid collection was feasible for only nine patients. Flow cytometry was performed on synovial fluid immune cell subpopulations in our previous study for a subset of five patients from whom sufficient synovial fluid volume could be obtained [8].

Statistical analyses

GraphPad Prism 6.0 (La Jolla, USA) and JMP Pro 17 (Cary, USA) software were used for statistical analyses and to create plots. Statistical tests are specified in the figure and table captions. Spearman's correlation was selected as a non-parametric statistical test to investigate associations between KOOS changes (delta or percentage change) and other variables, given that variables within our dataset violated assumptions of normality and homoscedasticity. P values derived from Spearman's correlation were not corrected for multiple comparisons given our exploratory analyses with a limited dataset, and requirements for normality to apply multiple comparison corrections. $P < 0.05$ was considered statistically significant.

RESULTS

Patient responder status

We systematically investigated multivariable factors affecting KOA patient responder status including 12-month patient-reported KOOS data previously reported [8]. We applied OMERACT-OARSI criteria [26] to determine responder status, with modifications as described in the Methods. Patients were classified as: i) Function-Pain Responders (exceeding thresholds in <u>both</u> ADL and Pain scores); ii) Function Responders (exceeding thresholds in ADL scores), iii) Pain Responders (exceeding thresholds in Pain scores); and iv) Non-Responders (exceeding thresholds in neither ADL nor Pain) (**Table 1**). Out of twelve study patients, we identified eight Responders and four Non-Responders. The eight Responders were sub-divided into six Function-Pain Responders (3/6 Function-Pain Responder patients self-identified as women); seven Function Responders (including the six Function-Pain Responders; 4/7 Function Responder patients self-identified as women); and seven Pain Responders (including the six Function-Pain Responders; 4/7 Pain Responder patients self-identified as women). The remaining four patients were classified as Non-Responders. While our sample size is limited, the binary responder/non-responder criteria are useful in determining factors affecting patient clinical responses. We include correlation analyses with all KOOS subscale scores to further supplement our analyses. We did not see dose-dependency and observed Function-Pain Responders across all three MSC(M) doses including the 1×10^6 dose (2/4 patients), 10×10^6 dose (1/4 patients), and the 50×10^6 dose (3/4 patients) (**Table 1**).

Responder vs. Non-Responder classification holds at 24-months and at longer 8–10-year follow-up

Patient follow-up data out to 24 months showed that patient responses were largely maintained (**Fig. 1**). Specifically, patients categorized at 12 months as Function-Pain Responders displayed significantly improved KOOS scores in all subscales at **both** 12- and 24-months follow-up, relative to baseline (**Fig. 1A**), supportive of persistent effects of autologous MSC(M) with a single injection. Patients categorized as Non-Responders at 12 months similarly showed no significant changes in each of the KOOS subscales or Overall KOOS at 12- and 24-months relative to baseline, indicating that the clinical phenotype features of this group neither improved nor worsened compared to baseline. No significant differences were observed when comparing outcomes at 12 versus 24 months for any KOOS subscale in both responder and non-responder patient groups.

Similar results were obtained when patients were categorized according to either Function-Responders (**Fig. 1B**) or Pain Responders (**Fig. S1A**); however, the improvements in Sports/Rec scores were observed only at the 12- and not the 24-month point relative to baseline for these groups.

We further analyzed patient follow-up at 8-10 years after MSC(M) injection, with KOOS scores obtained for 10 of 12 patients (**Fig. 1C, 1D**). Patients classified as Responders (at 12-months) maintained significant improvement in multiple KOOS subscales at 8-10 years follow-up (**Fig. S2**).

Of the eight 12-month responder patients (including Function-Pain Responders, Pain Responder, and Function Responder), seven patients maintained their responder status at 24 months. Of these seven patients, 5 maintained Responder status at 8-10 years, and one patient (who reported only that they underwent knee arthroplasty surgery) did not provide long-term KOOS data. Within this group of 12-month Responders, 8–10-year follow-up data showed that 2/8 patient received no additional interventions, 2/8 received injections including viscosupplementation or platelet-rich plasma, and 3/8 patients underwent knee arthroplasty surgery.

In terms of the four 12-month Non-Responder patients, all patients maintained Non-Responder status at 24 months, and one patient was lost to follow-up at the 8–10-year time point. The remaining three patients maintained Non-Responder status at 8-10 years, with 1/4 patients reporting no additional interventions and 2/4 patients reporting a knee injection for viscosupplementation.

MSC Batch Fitness Status

Gene panel discriminates between clinically effective and ineffective MSC(M) donor batches

Using biobanked MSC(M) samples cultured under pro-inflammatory licensing conditions, we measured expression of a putative immunomodulatory gene panel that we previously identified [20]. Gene expression was measured under pro-inflammatory licensing conditions to measure the immunomodulatory response of MSC(M), as has been widely demonstrated in the field [29]; MSC(M) injected intra-articularly would encounter synovial fluid, which contains pro-inflammatory mediators [30] that would similarly license the MSC(M).

Principal Component (PC) analysis revealed distinct clustering between MSC(M) donor batches labelled as Function-Pain Responders compared to Non-Responders along the PC1 axis (accounting for 29.3% of total variance), with higher expression of *CCN2* and lower expression of angiogenic markers *THBS1*, *CXCL8*, and *ANGPT1* contributing most strongly to higher PC1 scores (**Fig. 2A**, **Fig. S3A**). PC1 scores were significantly lower for Function-Pain Responders, Function Responders, and Pain Responders relative to Non-Responders (**Fig. 2B**, **Fig. S3B**), confirming the utility of the gene expression panel to distinguish MSC(M) derived from responders and non-responders. To evaluate model robustness, outlier analysis was performed by calculating T^2 statistic, identifying three outlier data points (two Responder, one Non-Responder); however, the PCA model returned similar results even with these data points removed with clear separation of responder/non-responder distributed across the expected eigenvectors (**Fig. S3C**).

Higher basal gene expression of the angiogenic gene, *ANGPT1* in 9 samples of MSC(M) strongly (Spearman's rho >0.68) and significantly negatively correlated to improvements in KOOS Pain, ADL, Sports/Rec, QOL, and Overall KOOS at 12 months, as well as to improvements in KOOS ADL at 24 months relative to baseline (**Table S3**). In contrast, higher basal levels of the mitogenic factor, *CCN2* in MSC(M) strongly (Spearman's rho >0.66) and significantly positively correlated to improvements in KOOS Pain, QOL, Other Symptoms, and Overall KOOS at 12 months relative to baseline. PC1 scores were indexed as a composite score for the basal immunomodulatory gene expression profile, and robust negative correlations were observed between PC1 scores and improvements in KOOS Symptoms, ADL, and Overall KOOS at both the 12- and 24-month time points relative to baseline, as well as KOOS QOL (12-month only) and KOOS Pain (24-month only) (**Fig. 2C**,

Table S3). We also investigated correlations between the PC1 score with changes in KOOS at 8-10 years follow-up and determined continued, significant negative correlations with KOOS ADL and Overall KOOS (**Table S3**).

To further evaluate the strength of these trends, we performed a leave-out-one sensitivity analysis in which correlations to the PC1 gene expression score were measured after omitting one patient at a time, focusing on changes in ADL, Pain, and Sports/Rec as key KOOS subscales (**Table S4**). For all Spearman's correlations to 12-month outcomes, the average p value across all leave-one-out correlations maintained significance. At 24 months, the correlation to percent change in ADL maintained significance, while the correlation to percent change in KOOS Pain was not significant. Previously significant Spearman's correlation with KOOS ADL at 8-10 years showed a reduced Spearman's rho value that was no longer significant. Taken together, these findings validate the utility and robustness of our previously identified gene panel [20] to discriminate clinical responsiveness to MSC(M).

Modulation of monocyte phenotype by donor MSC(M) *in vitro* correlates to patient responder status

We employed functional *in vitro* monocyte polarization read-outs using indirect co-culture of MSC(M) with mismatched human peripheral blood-derived monocytes to assess baseline MSC fitness as part of a matrix of CQAs (**Fig. 3A**). Discriminant analysis demonstrated distinct clustering and greater separation of monocyte phenotypic profiles when donor MSC(M) were classified as Function Responders versus Non-responders (**Fig. 3B**). Co-cultures of monocytes with Function Responder MSC(M) donor batches induced significantly upregulated expression of the inflammation-resolving markers *IL10* and *STAB1*, as well as reduced expression of *CD86* compared to Non-Responders (**Fig. 3B**). The immunosuppressive marker, *CD274*, was upregulated by MSC(M) donor batches classified as Non-Responders compared to Function Responders. Overall, MSC(M) donor batches classified as Function Responders modulated *in vitro* monocyte polarization toward a distinct and more pro-resolving phenotype. Discriminant analysis showed reduced separation when donor MSC(M) batches were classified according to Function-Pain Responder (**Fig. 3C**) or Pain Responder categorization (**Fig. S3D**) as indicated by greater misclassified data points and reduced Entropy R^2 values relative to the model using the Function Responder classification (**Table S5**).

Specific microRNAs distinguish responder donor MSC(M) batches

To probe unbiased baseline differences in responsive MSC(M) donor batches, we performed microRNA-Seq. PC analysis demonstrated that samples clustered by donor batch and responder group rather than by synovial fluid stimulation (**Fig. 4A**). We identified 33 significantly differentially expressed microRNAs between MSC(M) donor batches classified as Function Responders vs. Non-Responders after correction for multiple comparisons; 11 microRNAs were upregulated, and 22 microRNAs were downregulated in Function Responders (**Fig. 4B**, **Table S6**).

We focused our analysis on the microRNA profile of MSC(M) classified as Function Responders, conducting molecular interaction analysis on predicted gene targets for microRNAs that were significantly differentially expressed between Function Responder and Non-Responder MSC(M). Gene targets related to cell cycle (GTPases), phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), transforming growth factor beta (TGF-β), VEGF, SMAD proteins, apoptosis, and other pathways were identified (**Fig. 4C,D**; full list of pathways in **Suppl. File 2**). Gene ontology analysis identified biological processes linked to neuronal processes, histone modification, cell growth, ossification, Wnt signaling, and others (**Fig. 4E,F**; full list of pathways in **Suppl. File 2**). Taken together, our microRNA-sequencing data and analysis provide further characterization and potential mechanistic insights into MSC(M) therapeutic effects in KOA patients.

Baseline Patient/Disease Status Baseline clinical phenotype correlates with 12- and 24-month and 8–10-year improvements

We probed the relationship between baseline KOOS subscales with subsequent responder status (positive delta or percent change values indicate improvement) (**Fig. 5**). Patients classified as Function Responders displayed significantly elevated Overall KOOS and KOOS Sports/Rec at baseline compared to the Non-Responder group, supportive of more severe baseline clinical presentations in this group (**Fig. 5A**). Function-Pain Responders and Pain Responders displayed no significant differences in baseline relative to Non-Responders (**Fig. 5A and S1B**).

Next, we analyzed correlations between the baseline scores for all five KOOS subscales as well as Overall KOOS with changes in these scores at 12- and 24-months (**Table S7**). The baseline Sports/Rec scores positively and significantly correlated with improvements in: i) KOOS ADL at 12- and 24-month relative to baseline and in ii) KOOS ADL, iii) KOOS Symptoms, and iv) Overall KOOS at 24 months (**Fig. 5C**).

Given that the KOOS Sports/Rec subscale measures difficulty in activities associated with knee function [31], we further probed the relationship between the KOOS Sports/Rec score and physical activity using the MARS [32]. Correlation analyses demonstrated that higher baseline MARS scores (indicative of frequent physical activity) were significantly associated with lower (i.e., less severe) baseline KOOS Sports/Rec scores (**Fig. 5D**). Interestingly, a higher MARS score significantly correlated to lesser improvements (or worsening) in i) KOOS Symptoms, and ii) KOOS Pain at 12 months (**Fig. 5D**, **Table S8**). No significant correlations were observed for the 24-month timepoint.

Using only the baseline clinical phenotype features that correlated to significant improvements in KOOS at 12- or 24-months follow-up, we examined correlations of the baseline features to 8-10-year follow-up KOOS data. Only the baseline MARS score continued to have significant negative correlation with changes in KOOS Symptoms at 8-10 years follow-up (**Table S7, S8**).

Baseline synovial fluid biomarkers correlate to 3-, 12- and 24-month patient improvements

The levels of inflammatory, angiogenic, metabolic and cartilage extracellular matrix-associated biomarkers in synovial fluid were previously reported [8]; however, we analyzed correlations of biomarker levels at baseline with 12-, 24-, and 8–10-year KOOS improvements (**Tables S9, S10**).

Correlation analyses revealed several baseline synovial fluid biomarkers that significantly correlated with improvements in KOOS Sports/Rec scores at 12 and 24 months. We observed one significant **positive** correlation for an adipokine, resistin, with greater improvements (delta values) in Sports/Rec scores at 24 months relative to baseline (r_s of 0.72; **Fig. 6A**), while all other significant correlations for inflammatory, chemotactic, and angiogenic factors were **negatively** correlated with improvements in PROMs at follow-up time points. Specifically, lower levels of TIMP1 significantly negatively correlated to percentage changes in KOOS Sports/Rec scores at 24 months relative to baseline (r_s of -0.073). CCL2 significantly negatively correlated with changes in KOOS Sports/Rec scores at 24 months relative to baseline (r_s of -0.72, -0.85). Significant negative correlations were also observed between changes in KOOS Pain at 12 months relative to baseline with the levels of the pleiotropic HGF (r_s of -0.79, -0.71), as well as the potent angiogenic factor VEGFA (r_s of -0.81) **Fig. 6B**).

Baseline synovial fluid levels of soluble (s)CD14 and CCL2 significantly and negatively correlated with improvements in KOOS Symptoms and Sports/Rec scores at 3 months relative to baseline (**Fig. 6C**, **Tables S11, S12**); 3 months was the earliest timepoint when patients displayed improvements in PROM [8]. The covariance matrix for local biomarkers that showed significant correlations is reported in **Table S13**. Briefly, TIMP1, resistin, HGF and VEGFA covary with each other, while CCL2 and sCD14 covary with each other, indicative of multiple pathways that may influence MSC(M)-mediated clinical responsiveness.

Using only the synovial fluid biomarkers that showed significant correlations to improvements in KOOS at 12 or 24 months, we performed correlations to changes in KOOS at 8-10 years follow-up but observed no significant correlations (**Table S9, S10**).

Additionally, we performed the leave-out-one sensitivity analysis in which 12- and 24-month correlations to local biomarkers were measured, omitting one patient at a time (**Table S14**). For correlations performed using 12-month outcomes, the average p value across all leave-one-out correlations maintained significance for HGF with KOOS Pain (delta only but not percent change) and for VEGF with KOOS Pain (percent change). At 24 months, the average correlations between resistin and KOOS Sports/Rec (delta) as well as between CCL2 and KOOS Sports/Rec (percent change) maintained significance. The average correlations between CCL2 and KOOS Sports/Rec (delta) as well as between TIMP1 and KOOS Sports/Rec (percent change) were not significant.

Baseline synovial fluid immune cell phenotype correlates with responder/non-responder classification

We also analyzed synovial fluid immune cell subpopulations at baseline using data generated in our previous study [8]. While this analysis was feasible for only five patients due to limited synovial fluid volumes, our data demonstrates proof-of-concept for probing baseline levels of $CD14^{+}CD16^{+}$ and $CD14^{+}CD163^{+}$ monocyte/macrophages (MΦs) (**Fig. S4A**) as well as $CD3^{+}CD4^{+}CD69^{+}$ and $CD3^{+}CD4^{+}CD25^{+}$ T helper cells (**Fig. S4B**) as an important baseline immune marker of patient disease status and molecular endotype; higher frequencies of Mϕs and activated T cells are present in non-responders at baseline, although not significant given the limited sample size.

Specific baseline systemic biomarker levels correlate with 3- and 6-month but not 12- and 24-month patient improvements

We further measured levels of systemic inflammatory biomarkers (sCD163; C-reactive protein, CRP; S100A8/A9; tumor necrosis factor-alpha, TNFα; and interleukin (IL)-6) and systemic biomarkers of cartilage breakdown (serum C1-2C, plasma COMP and HA, and urine CTX-II and C2C-HUSA were previously measured [8]) in biobanked serum and plasma of all twelve patients at baseline (**Table S15, S16, S17**). Higher levels of baseline plasma TNFα significantly positively correlated to the percentage change in KOOS Pain and QOL improvement at 6 months (**Fig. S5A**, **Table S17**). On the other hand, baseline serum levels of the alarmin S100A8/A9 significantly negatively correlated to changes in KOOS QOL, and Sports/Rec at 3 months relative to baseline (**Fig. S5B**, **Table S17**). Systemic levels of biomarkers for cartilage breakdown showed no significant correlations to changes in KOOS at any time points. Systemic biomarker levels thus showed limited sensitivity with correlations to short-term PROM improvements only. Altogether, there was no discernible baseline profile of systemic biomarkers to prospectively identify responder KOA patients.

DISCUSSION

Statistically powered clinical trials using MSCs have reported conflicting results in KOA patients [5,12]. To address underlying factors that dictate responsiveness to MSCs, we analyzed our test clinical dataset [8] and demonstrated specific MSC(M) donor batch characteristics and responsive KOA patient features that correlated with improvements in PROMs at 12 and 24-months that were sustained out to 8-10 years, in 62.5% of responders and 100% of non-responders (including patients lost to follow-up). We anticipate that this combinatorial analysis will serve as an integrated algorithm, that can be tested in future prospective KOA clinical trials (and retrospectively on previous trial datasets) to i) screen potent donor/batches of MSC; ii) to identify responsive recipients. Our data shows that MSC(M) donor batches that are responsive have high basal immunomodulatory fitness as measured by reduced levels of angiogenic genes, higher monocyte modulatory activity and distinct signature of microRNAs. In parallel, KOA patients most responsive to MSC(M) donor batches had more severe baseline disease; reduced knee functionality associated with sports and recreation activities; reduced physical activity; reduced levels of soluble angiogenic factors in the local joint environment and trends pointing to lower baseline levels of intermediate monocyte/macrophages and activated T cells. We used a previous clinical trial [8], where we manufactured 12 distinct, patient-specific lots of MSC(M).

While we acknowledge the limited sample size, our clinical trial was data-rich with multiple baseline biomarker and clinical readouts and offered an excellent test dataset to study batch variations as we manufactured twelve patient-specific batches of autologous MSC(M). Leave-out-one sensitivity analysis confirmed the robustness of correlations with MSC(M) fitness genes panel and baseline synovial fluid biomarkers mitigating concerns regarding the robustness of the correlations using a small sample size.

Additionally, we provide longitudinal 12-, 24-month and 8–10-year follow-up data and are one of the first groups to report such long-term clinical data. Responders showed significant improvements in PROMs at both 12- and 24- months relative to baseline and significant sustained responder and non-responder status was maintained to 8-10 years. We note that 2/8 (25%) Responder and 2/4 (50%) Non-Responder patients received additional injections over the intervening years, and 3/8 Responders received or were awaiting a total knee replacement. Additionally, 3/8 Responders (37.5%) and 1/4 Non-Responders (25%) did not receive additional interventions. The higher number of total knee replacements in the Responder group may be related to differences in sample size (N=8 Responders vs N=4 Non-Responders). Given the extended lag in the follow-up period, it is not possible to confirm if the single MSC(M) injections delayed knee replacements to this 8–10-year follow-up window. We also cannot rule out the effects of additional interventions that may have contributed to the PROM data obtained beyond the study endpoint of one year in both Responder and Non-Responder patient groups [5,12].

KOA patients classified as Function Responders showed greater correlation to MSC(M) donor potency fitness and a more distinct baseline clinical phenotype (especially KOOS Sports/Rec scores). This is interesting given that immunomodulatory therapies may be expected to induce greater effects on patient pain [33]; however, MSCs act through multimodal mechanisms and both pain and functional improvements are commonly documented [34]. KOA patients with higher disease severity, especially reduced levels of high-level knee functionality had greater improvements in daily functioning of the knee (KOOS ADL), confirming inter-relationships between these KOOS ADL and Sports/Rec subscales [31]. We also observed that patients with higher baseline MARS scores had lesser improvements in pain and other symptoms, suggesting that patients who may have persisted through pain in physical activity may have aggregated progression. [31]. The demographics of the patient cohort study with an age range of 40-65 years, body mass index (BMI) <30,

relatively neutral knee alignment, and KL grade 3 or 4 KOA provide clues to the utility of the KOOS Sports/Rec subscale [34–36]. Notably, our analysis demonstrating patients with more severe KOA were more responsive to MSC(M) treatment aligns with findings in acute graft-versus-host disease [37], myocardial infarction [38] and ulcerative colitis [39], where more severe disease patients respond better to MSC treatments.

We focused on basal immunomodulatory fitness of MSC(M), especially their ability to modulate monocytes, given their importance as a clinically relevant mechanism of action in KOA [4,7,8]. Our previously identified immunomodulatory gene panel for MSC(AT) [20] successfully distinguished MSC(M) donors classified under all categories of Responders versus Non-Responders. These differences were robust; dimension-reduced PC1 scores capturing multivariate gene expression profile were strongly and significantly correlated inversely to patient improvements at 12-, 24-months and even 8–10-year follow-up and are thus more reliable than the use of individual genes. Leave-out-one sensitivity analysis also confirmed the robustness of correlations to dimension reduced PC1 scores at 12-months, assuring the individual donors were not skewing the correlation values. Notably, this panel includes predominantly angiogenic markers that were previously inversely correlated to monocyte polarization toward inflammation-resolving phenotypes [20]. The data also supports other work indicating inverse relationships between angiogenic and immunomodulatory fitness of MSC(M) [40,41]. While the transcriptome of MSC(AT) and MSC(M) are distinct [42], the putative immunomodulatory genes we identified for MSC(AT) are valid for MSC(M) as well, speaking to their broad utility to serve as MSC CQAs. Our immunomodulatory gene panel aligns with expression of another immunomodulator, indoleamine 2,3-dioxygenase (IDO) that correlates with in autologous infrapatellar fat pad-derived adipose tissue MSC(AT) functional improvements in KOA patients [7]. These data support our hypothesis that MSCs with high basal immunomodulatory fitness produce greater improvements in knee function. Nonetheless, the use of autologous MSCs in both studies [7,8] adds a layer of complexity, given that systemic factors such as age, BMI, and other health conditions were dually accounted for in both the basal fitness of the cells [43,44], as well as baseline patient disease factors [45]; the interplay between these different factors is likely to have contributed to the overall responsiveness.

MSC(M) derived from Responders modulated $CD14^+$ monocytes to more pro-resolving subtypes *in vitro*. Monocytes represent major immune effector cells modulated by MSC(M)

injection in KOA [8,46]. While we showed preferential upregulation of pro-resolving $CD14^+$ monocyte phenotype we also observed upregulation of markers typically associated with pro-inflammatory phenotypes. Reliance on dichotomized gene expression markers is not indicative of the plurality of monocyte functionality [47]. Rabani *et al*. demonstrated that induction of a mixed phenotype of monocytes may be important for MSC-mediated microbicidal functions within a sepsis model [48]. We used LPS after co-culture with MSC(M) to elicit stronger gene expression readouts from the $CD14^+$ monocytes; LPS is present in synovial fluid of KOA patients and its levels correlate to increased disease severity [49], justifying its inclusion in our assay. Nonetheless, the distinct polarizing ability of Function Responder MSC(M) donors vs. Non-Responder donors offers another readout to prospectively screen potent MSC donor/batches.

MSC(M) from Function or Function-Pain Responders had a significantly different microRNA signature compared to Non-Responders. MicroRNAs are important epigenetic regulators of MSC fitness [50] and can mediate multiple MSC mechanisms of action [51]. Pathways related to inflammation, fibrosis, angiogenesis, and ossification, relevant to KOA pathophysiology were identified. We also identified enrichment of pathways involved in cell cycle, in line with RNA-sequencing and cell proliferation analyses performed on autologous MSC(AT) used in KOA [52]. Surprisingly, our analysis identified enrichment of several neuronal processes; however, given the role of innervation and neuronal signaling in joint pain in KOA [53], these microRNAs may be implicated in mediating analgesic effects of MSC(M). The functionality of the microRNAs require further study, but these differentially expressed panels may also serve as additional prognosticating CQAs for identifying basally fit MSC(M) in treating KOA patients.

Responders to MSC(M) injection displayed a mixed signature of higher baseline synovial fluid levels of an adipokine (resistin), and lower levels of inflammatory, angiogenic, and extracellular matrix-associated biomarkers, including TIMP1, CCL2, HGF, VEGFA, and sCD14, accompanied by lower levels of intermediate $CD14^+CD16^+$ monocyte/macrophages (Mϕs). While increased levels of resistin in synovial fluid may be linked to higher levels of inflammation in KOA [54], lower levels of HGF, TIMP1, VEGFA, CCL2, and sCD14 indicate lower levels of active local inflammation, immune cells and angiogenesis [55–58]. Low levels of sCD14 have been previously correlated to lower levels of activated neutrophils and macrophages in KOA [56] and to lower levels of $CD14^+CD16^+$ intermediate synovial

fluid MΦs[46]. We have also previously reported reduction in the levels of $CD14^{+}CD16^{+}$ intermediate synovial fluid MΦs after MSC(M) treatment [8], corroborating other datasets showing reduced MΦ levels post-MSC treatment [39,59]. However, synovial fluid collection and local biomarker measurement was possible for only a subset of patients who had active disease presentation, and thus likely had increased inflammation and greater joint pain [60,61], suggestive of a selection bias of patients who had active synovitis. These KOA patients thus likely represent an inflammatory phenotype with active disease presentation, and responsiveness to MSC(M) treatment. There is emerging data showing distinct immunophenotypic classification of KOA patients based on activated levels of MΦs and T cells [62], in line with our preliminary data that needs further study.

There were only short-term significant, albeit mixed correlations between systemic biomarkers such as TNFα (in plasma) and S100A8/A9 (in serum) which positively and negatively correlated to PROM improvements, respectively. Higher circulating levels of both TNFα and S100A8/A9 may correlate to greater disease severity and systemic inflammation [54,56]. Interestingly, a similar mixed signature of systemic inflammatory biomarkers measured at baseline has been reported in responders versus non-responders to intravenous infusion of allogeneic Wharton's jelly-derived MSCs in rheumatoid arthritis patients [61]. Baseline serum CRP levels, an inflammatory biomarker in heart failure, chronic obstructive pulmonary disease, and other inflammatory conditions [62] failed to correlate with improvements in KOOS at all time points as did cartilage breakdown biomarkers, previously shown to correlate with worsening KOA pain [63]. Overall, our analyses suggest that local biomarkers provide improved sensitivity compared to systemic biomarkers [24,55].

Limitations include small sample size, which we addressed with various sensitivity analyses and the absence of a control group, although effects were measured at multiple timepoints including 12, 24 and 8-10 years, relative to baseline. Given that KOA is a progressive condition [64], approximately 30% of patients display rapidly worsening of PROMs over two years leading up to joint surgery [65]. Although we cannot rule out placebo effects, we saw less than 30% of patients who reported worsening outcomes, potentially speaking to a treatment effect. Indeed, analysis of Non-Responders using the rigorous OMERACT-OARSI criteria [26] showed that even patients within this category did not show any significant worsening in PROMs over two years and 8-10 years of follow-ups. We cannot however rule out the effects of other interventions or the absence of intervention on the long-term PROM

scores, but do report that 62.5% of Responders maintained Responder status while 100% of the Non-Responders maintained Non-Responder status [5,12].

CONCLUSIONS

We have demonstrated that clinical batches of MSC(M) that resulted in positive clinical outcomes differ significantly in specific measurable attributes compared to MSC(M) batches that failed to improve pain or function. These quantitative, multivariate, and functionally relevant attributes can define CQAs for MSC(M) that can be evaluated to strategically design future KOA trials to ensure higher success rates of MSC injection. Furthermore, we correlated baseline clinical features and synovial fluid biomarkers to responder status in KOA patients after MSC(M) injection. These analyses may be applied retrospectively to recently published KOA clinical trials investigating MSC(M) [12] and umbilical cord-derived MSC [5] injections.

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Disclosure of Potential Conflicts of Interest

Kevin P. Robb declares current employment status with STEMCELL Technologies. Sowmya Viswanathan declares 60% ownership of Regulatory Cell Therapy Consultants Inc., which does not conflict with this paper in any way. Sowmya Viswanathan, Razieh Rabani, and Kevin P Robb are co-inventors on patent applications filed with UHN based on this work. The remaining authors declare they have no competing interests.

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Table 1. Patients classified as Function-Pain Responders, Function Responders, Pain Responders, or Non-Responders based on changes in KOOS ADL and Pain scores at 12 months relative to baseline. Function-Pain Responders (N=6 patients; bolded) were designated based on a delta value ≥ 10 and percentage change ≥ 20% in both KOOS pain and ADL scores based on OMERACT-OARSI criteria [26]. Function Responders (N=1; italics) and Pain Responders (N=1; italics) were classified according to the same thresholds for patients responding in either function (ADL) or pain scores, respectively. ADL: function in daily living; KOOS: knee injury and osteoarthritis outcome score.

				Pain		**ADL**	
Response classification	**Subject ID**	**Cell dose (x 10^6 cells)**	**Patient Gender**	**Delta**	**Percent Change**	**Delta**	**Percent Change**
Non-Responder	1	1	Man	5.50	28.35	2.90	32.95
Non-Responder	2	1	Man	11.10	18.17	-5.90	-25.11
Function-Pain Responder	**3**	**1**	**Woman**	**16.60**	**49.85**	**25.00**	**80.91**
Non-Responder	4	10	Man	-2.70	-48.21	0.00	0.00
Function-Pain Responder	**5**	**10**	**Man**	**13.90**	**29.45**	**11.80**	**100.00**
Function-Pain Non-Responder/ Pain Responder	*6*	*10*	*Woman*	*16.70*	*33.40*	*-8.80*	*-200.00*
Function-Pain Responder	**7**	**50**	**Woman**	**30.60**	**100.00**	**20.60**	**100.00**
Function-Pain Responder	**9**	**50**	**Woman**	**22.20**	**31.99**	**19.10**	**43.31**
Function-Pain Non-Responder/ Function Responder	*10*	*50*	*Woman*	*11.10*	*19.04*	*17.70*	*42.96*
Function-Pain Responder	**11**	**50**	**Man**	**36.10**	**81.31**	**20.60**	**70.07**
Non-Responder	12	10	Man	-30.50	-78.41	-14.70	-45.37
Function-Pain Responder	**13**	**1**	**Man**	**38.90**	**63.67**	**39.70**	**77.09**

Figure Legends

Fig. 1. Osteoarthritis patient responders demonstrate improvements in KOOS subscales that are maintained out to 24 months follow-up post-injection. A and B) Patients categorized as Function-Pain Responders (A) and Function Responders (B) displayed significant improvements (indicated by lower score values) in KOOS subscales and overall KOOS at 12- and 24-months follow-up relative to baseline, while Non-Responders displayed no significant changes. KOOS scales were inverted such that higher scores represent greater knee-related issues. C) Flow chart summary of patient responder status at 12-months, 24-months, and 8-10-years follow-up. D) Summary of OARSI-OMERACT Responder classification in 2025 after 8-10 years of follow-up. Changes in patient responder status at 8-10 years are bolded. Patient-reported follow-up notes on additional interventions are included in the right column. N=12 patients. Two-way repeated measures ANOVA, Dunnett's multiple comparisons test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; asterisks indicate significant differences for responder group relative to baseline at the time points indicated. ADL: function in daily living; Sports/Rec: function in sport and recreation; QOL: knee-related quality of life; KOOS: knee injury and osteoarthritis outcome score; PRP: platelet-rich plasma; TKR: total knee replacement.

Fig. 2. Independent validation that a previously identified gene panel distinguishes clinically effective MSC(M) donors from non-responder MSC(M) donors. A) Principal component (PC) analysis of MSC(M) expression of an immunomodulatory gene panel identified previously for MSC(AT) (*TNFAIP6*, *ACTA2*, *ANGPT1*, *CXCL8*, *EDN1*, *THBS1*, *PDCD1LG2*, *CCN2*; [20]) expressed under licensed conditions. The corresponding loading plot of eigenvectors (right) indicates the relative contribution of each gene to the PC1 and PC2 axes. B) MSC(M) derived from Function-Pain Responders and Function Responders displayed significantly lower PC1 scores relative to non-responders, indicating that the gene panel sensitively distinguishes MSC(M) from donors classified as responders. Student's t-test, ***$p<0.001$, ****$p<0.0001$. Horizontal lines: group mean; error bars: standard deviation. C) Significant negative correlations between PC1 scores and the percentage change at 24-months relative to baseline for KOOS ADL, pain, symptoms, and overall KOOS. Percent change in KOOS values were made relative to baseline and calculated such that positive values represent improvement. Spearman's correlation. N=9 MSC(M) donors; n=2-3 replicates/donor.

Fig. 3. *In vitro* monocyte/macrophage polarization readouts distinguish donor MSC(M) immunomodulatory fitness for responders versus non-responders and can be used to empirically rank the fitness of individual MSC(M) donors. A) Schematic of *in vitro* monocyte/macrophage (MΦ) polarization experiment. Indirect co-cultures of MSC(M) with human peripheral blood-derived MΦ was performed for 48 h prior to removal of MSC(M) and addition of lipopolysaccharide (LPS) for 4 h incubation. B and C) Summary of MΦ gene expression after co-culture with Function-Pain Responders (B) and Function Responders (C). Box-and-whisker plots (left) display changes in gene expression relative to MΦ cultured alone (Solo; dotted line). Horizontal line: median; hinges: first and third quartiles; whiskers: range. Two-way ANOVA, Tukey's post-hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ relative to Solo condition or to groups indicated by brackets. Discriminant canonical

plots (right) of -ddCt values relative to Solo condition. Inner ellipses indicate 95% confidence for the mean of each group; outer ellipses indicate the normal region estimated to contain 50% of the population for each group.

Fig. 4. microRNA-sequencing reveals microRNA signatures in Function Responder donor MSC(M). A) Principal component analysis of microRNA expression profiles demonstrate clustering in Function Responders versus Non-Responders. MSC(M) samples cluster by donor and responder status rather than by synovial fluid (SF) stimulation. B) Volcano plot displays 33 significantly differentially expressed microRNAs in MSC(M) derived from donors classified as Function Responders versus Non-Responders. C&D) Top 30 pathways identified through Reactome analysis of predicted gene targets for significantly upregulated (C) and downregulated (D) microRNAs. E&F) Top 30 pathways identified through Gene Ontology (Biological Processes) analysis of predicted gene targets for significantly upregulated (E) and downregulated (F) microRNAs.

Fig. 5. More severe KOOS and lower MARS physical activity scores correlate to improved patient-reported outcomes at 12- and 24-months follow-up. A) Patients categorized according to Function Responders displayed significantly more severe overall KOOS and Sports/Rec scores at baseline relative to Non-Responders. B) Patients categorized according to Function-Pain Responders and Non-responders displayed no significant differences between overall KOOS and KOOS subscales at baseline. Horizontal lines: group mean; error bars: standard deviation. C) Significant correlations between baseline KOOS Sports/Rec scores and improvements in delta ADL scores at 12 months relative to baseline, percentage change in symptoms at 24 months relative to baseline, delta ADL at 24 months relative to baseline, and delta overall KOOS at 24 months relative to baseline. D) Baseline MARS significantly correlated to baseline Sports/Rec, percent change in KOOS Symptoms at 12 months relative to baseline, as well as both delta and percent change in KOOS Pain at 12 months relative to baseline. KOOS scales were inverted such that higher scores represent greater knee-related issues; delta and percent change in KOOS values were made relative to baseline and calculated such that positive values represent improvement. N=12 patients. Student's t-test (for baseline Overall KOOS), Two-way ANOVA, Sidak's post-hoc test (for baseline KOOS Subscales), Spearman's correlation. *$p<0.05$, **$p<0.01$. n.s.; non-significant. ADL: function in daily living; Sports/Rec: function in sport and recreation; QOL: knee-related quality of life; Pt.: patient ID; MO: month; KOOS: knee injury and osteoarthritis outcome score; MARS: Marx Activity Rating Scale.

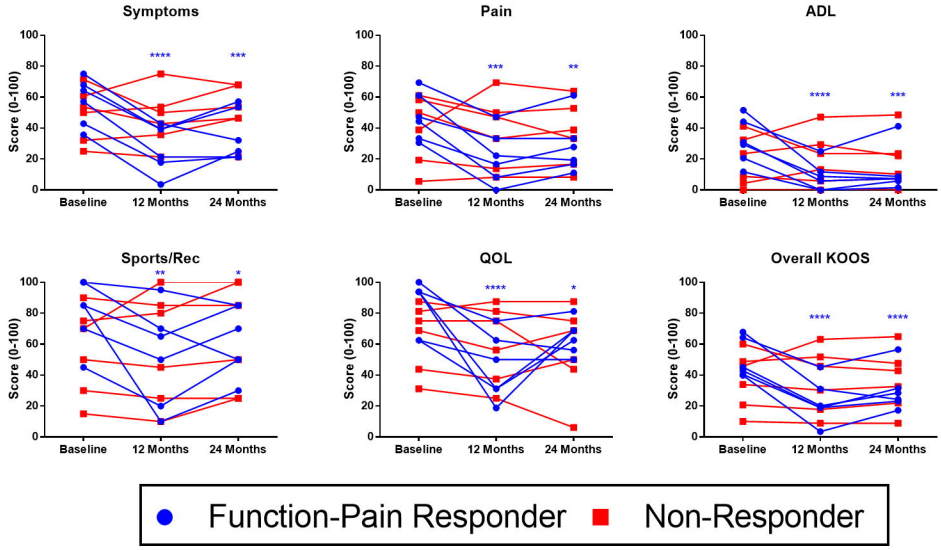
Fig. 6. Local synovial fluid biomarkers measured at baseline correlate to patient-reported outcome measures at 3, 12, and 24 months relative to baseline. A) Higher levels of resistin significantly positively correlated to delta values in KOOS Sports/Rec scores at 24 months. Higher levels of TIMP1 and CCL2 significantly correlated inversely to the percentage change and/or delta in KOOS Sports/Rec scores at 24 months relative to baseline. B) Higher levels of HGF and VEGFA in synovial fluid significantly correlated inversely to delta values and/or percentage change in KOOS Pain scores at 12 months relative to baseline. C) Higher levels of sCD14 and

CCL2 significantly correlate inversely to the percentage change and/or delta in KOOS Symptom and Sports/Rec scores at 3 months relative to baseline. Delta and percent change in KOOS values were made relative to baseline and calculated such that positive values represent improvement. N=8-9 patients. Spearman's correlation. Sports/Rec: function in sport and recreation; MO: month; KOOS: knee injury and osteoarthritis outcome score; TIMP1: tissue inhibitor of metalloproteinases 1; CCL2: chemokine C-C motif ligand 2; HGF: hepatocyte growth factor; sCD14: soluble CD14; VEGFA: vascular endothelial growth factor A.

FIG. 1

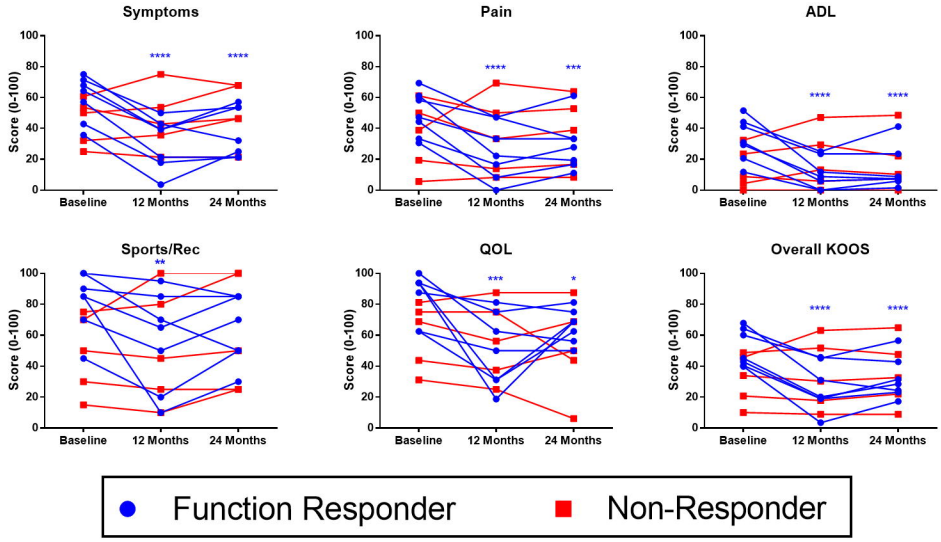
A

Function-Pain Responders

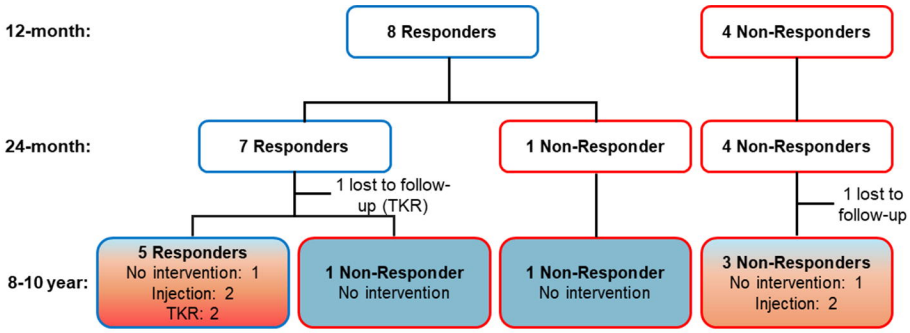


B

Function Responders



C



D

					8-10 year follow up scores				
					KOOS Pain		KOOS ADL		
Subject ID	Responder Class at 12 Months	Responder Class at 24 Months	Responder Class at 8-10 years	# Years Follow-up in 2025	Delta	% Change	Delta	% Change	Follow-up notes
1	Non-Responder	Non-Responder	Non-Responder	9 years, 10 months	-0.04	-0.21	-1.49	-16.93	Injection of viscosupplementation in 2024 and 2025
2	Non-Responder	Non-Responder	N/A	-	-	-	-	-	N/A
3	Function-Pain Responder	Function Responder	Function-Pain Responder	9 years, 6 months	30.52	91.65	27.96	90.49	PRP injection in 2023
4	Non-Responder	Non-Responder	Non-Responder	9 years, 4 months	-44.40	-792.86	-30.88	-3088.00	No surgical intervention
5	Function-Pain Responder	Pain Responder	N/A	-	-	-	-	-	TKR in 2025
6	Pain Responder	Pain Responder	Pain Responder	9 years, 0 months	11.11	22.22	-22.07	-501.59	Awaiting bilateral knee replacement
7	Function-Pain Responder	Function-Pain Responder	**Non-Responder**	8 years, 11 months	0.04	0.13	-1.46	-7.09	No intervention post injection
9	Function-Pain Responder	Non-Responder	**Non-Responder**	8 years, 6 months	-2.82	-4.06	-19.14	-43.40	No intervention post injection
10	Function Responder	Function-Pain Responder	Function-Pain Responder	8 years, 6 months	19.41	33.29	20.61	50.02	Viscosupplementation - no majory surgery
11	Function-Pain Responder	Function-Pain Responder	Function-Pain Responder	8 years, 6 months	24.96	56.22	16.16	54.97	No knee surgery/scope, no knee injection
12	Non-Responder	Non-Responder	Non-Responder	8 years, 4 months	-30.54	-78.51	-24.95	-77.01	Viscosuppementation in 2019
13	Function-Pain Responder	Function-Pain Responder	Function-Pain Responder	8 years, 2 months	49.99	81.82	39.74	77.17	TKR in 2025

FIG. 2

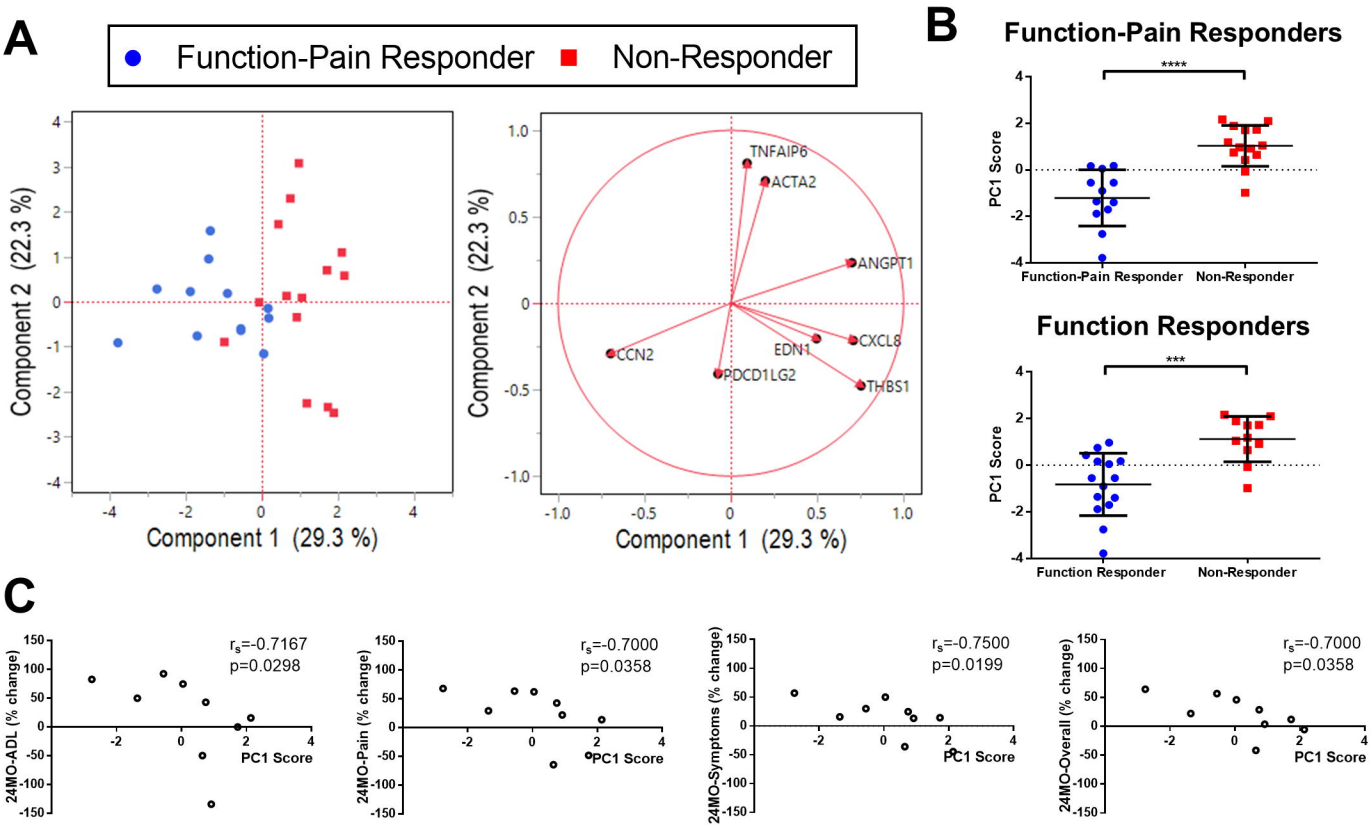

A
Function-Pain Responder
Non-Responder
Component 2 (22.3 %)
Component 1 (29.3 %)
TNFAIP6
ACTA2
ANGPT1
CXCL8
EDN1
CCN2
PDCD1LG2
THBS1
B
Function-Pain Responders
****
PC1 Score
Function-Pain Responder
Non-Responder
Function Responders
***
Function Responder
Non-Responder
C
24MO-ADL (% change)
r_s=-0.7167
p=0.0298
24MO-Pain (% change)
r_s=-0.7000
p=0.0358
24MO-Symptoms (% change)
r_s=-0.7500
p=0.0199
24MO-Overall (% change)
r_s=-0.7000
p=0.0358
PC1 Score

FIG. 3

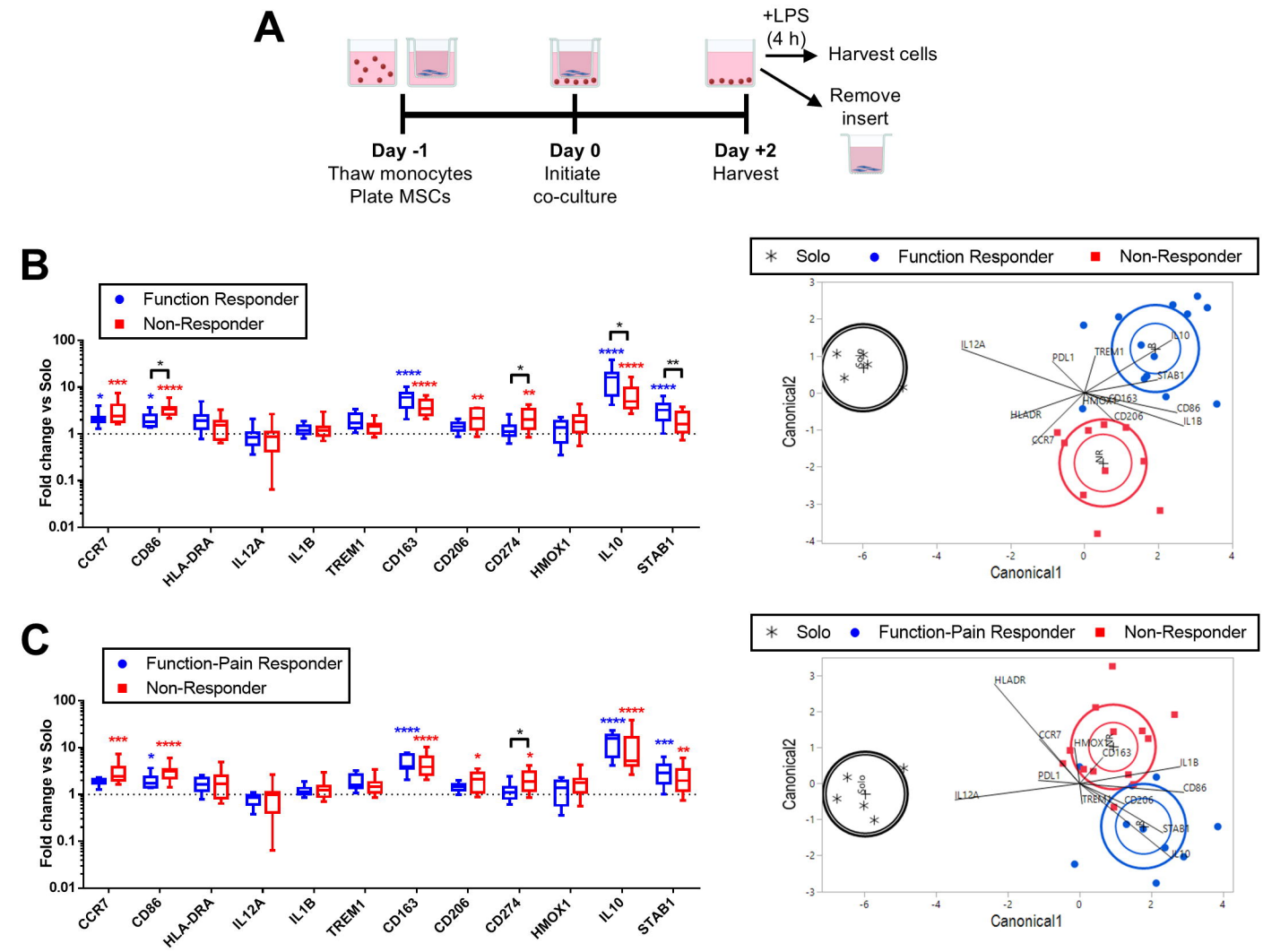

A
+LPS
(4 h)
Harvest cells
Remove
insert
Day -1
Thaw monocytes
Plate MSCs
Day 0
Initiate
co-culture
Day +2
Harvest
B
Function Responder
Non-Responder
Fold change vs Solo
CCR7
CD86
HLA-DRA
IL12A
IL1B
TREM1
CD163
CD206
CD274
HMOX1
IL10
STAB1
Solo
Function Responder
Non-Responder
Canonical1
Canonical2
C
Function-Pain Responder
Non-Responder
Fold change vs Solo
Solo
Function-Pain Responder
Non-Responder
Canonical1
Canonical2

FIG. 4

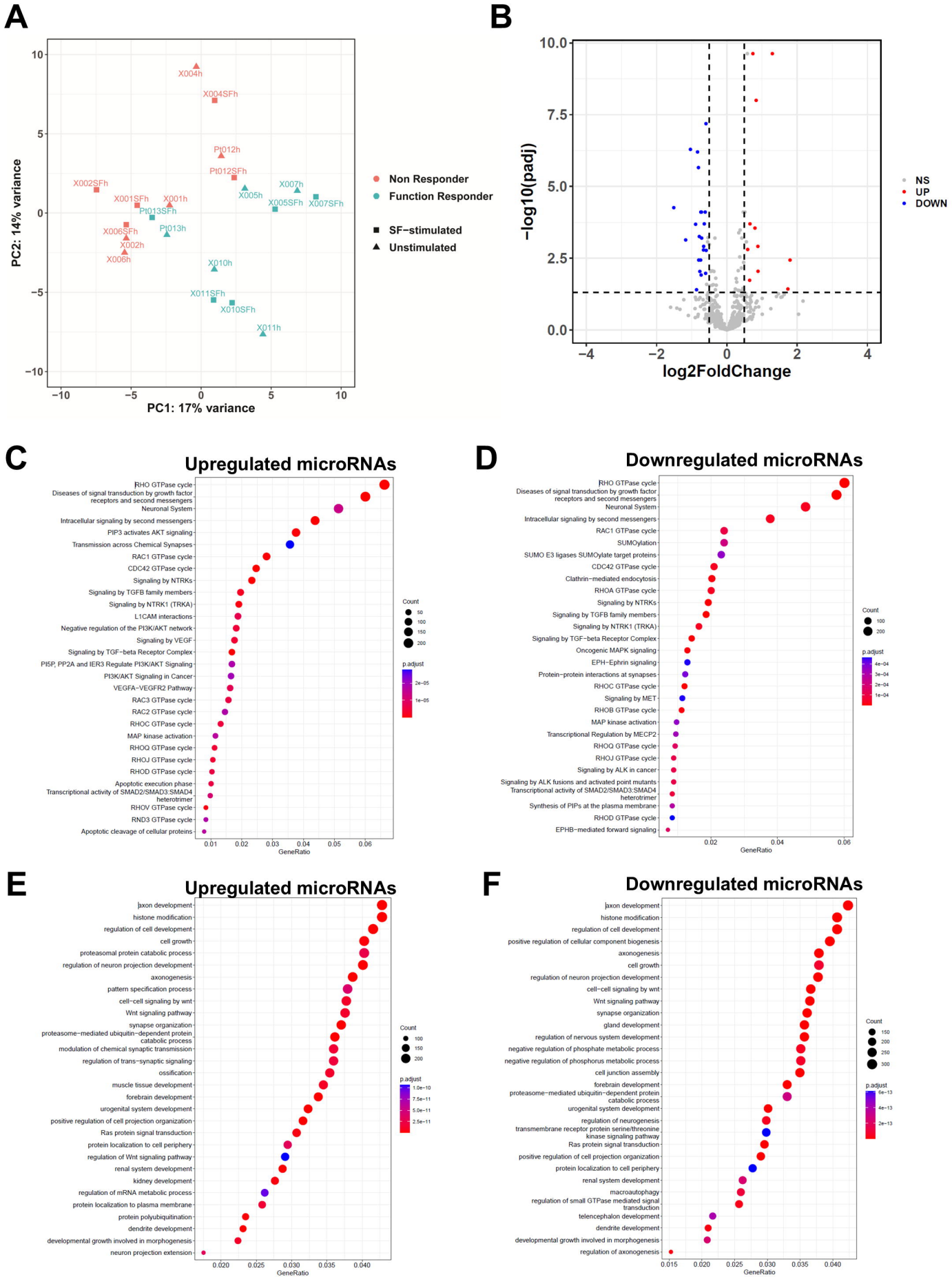

A
PC2: 14% variance
PC1: 17% variance
X004h
X004SFh
Pt012h
Pt012SFh
X002SFh
X001SFh
X001h
Pt013SFh
X006SFh
Pt013h
X002h
X006h
X005h
X007h
X005SFh
X007SFh
X010h
X011SFh
X010SFh
X011h
Non Responder
Function Responder
SF−stimulated
Unstimulated
B
−log10(padj)
log2FoldChange
NS
UP
DOWN
C
Upregulated microRNAs
RHO GTPase cycle
Diseases of signal transduction by growth factor receptors and second messengers
Neuronal System
Intracellular signaling by second messengers
PIP3 activates AKT signaling
Transmission across Chemical Synapses
RAC1 GTPase cycle
CDC42 GTPase cycle
Signaling by NTRKs
Signaling by TGFB family members
Signaling by NTRK1 (TRKA)
L1CAM interactions
Negative regulation of the PI3K/AKT network
Signaling by VEGF
Signaling by TGF−beta Receptor Complex
PI5P, PP2A and IER3 Regulate PI3K/AKT Signaling
PI3K/AKT Signaling in Cancer
VEGFA−VEGFR2 Pathway
RAC3 GTPase cycle
RAC2 GTPase cycle
RHOC GTPase cycle
MAP kinase activation
RHOQ GTPase cycle
RHOJ GTPase cycle
RHOD GTPase cycle
Apoptotic execution phase
Transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer
RHOV GTPase cycle
RND3 GTPase cycle
Apoptotic cleavage of cellular proteins
GeneRatio
Count
p.adjust
D
Downregulated microRNAs
RHO GTPase cycle
Diseases of signal transduction by growth factor receptors and second messengers
Neuronal System
Intracellular signaling by second messengers
RAC1 GTPase cycle
SUMOylation
SUMO E3 ligases SUMOylate target proteins
CDC42 GTPase cycle
Clathrin−mediated endocytosis
RHOA GTPase cycle
Signaling by NTRKs
Signaling by TGFB family members
Signaling by NTRK1 (TRKA)
Signaling by TGF−beta Receptor Complex
Oncogenic MAPK signaling
EPH−Ephrin signaling
Protein−protein interactions at synapses
RHOC GTPase cycle
Signaling by MET
RHOB GTPase cycle
MAP kinase activation
Transcriptional Regulation by MECP2
RHOQ GTPase cycle
RHOJ GTPase cycle
Signaling by ALK in cancer
Signaling by ALK fusions and activated point mutants
Transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer
Synthesis of PIPs at the plasma membrane
RHOD GTPase cycle
EPHB−mediated forward signaling
GeneRatio
Count
p.adjust
E
Upregulated microRNAs
axon development
histone modification
regulation of cell development
cell growth
proteasomal protein catabolic process
regulation of neuron projection development
axonogenesis
pattern specification process
cell−cell signaling by wnt
Wnt signaling pathway
synapse organization
proteasome−mediated ubiquitin−dependent protein catabolic process
modulation of chemical synaptic transmission
regulation of trans−synaptic signaling
ossification
muscle tissue development
forebrain development
urogenital system development
positive regulation of cell projection organization
Ras protein signal transduction
protein localization to cell periphery
regulation of Wnt signaling pathway
renal system development
kidney development
regulation of mRNA metabolic process
protein localization to plasma membrane
protein polyubiquitination
dendrite development
developmental growth involved in morphogenesis
neuron projection extension
GeneRatio
Count
p.adjust
F
Downregulated microRNAs
axon development
histone modification
regulation of cell development
positive regulation of cellular component biogenesis
axonogenesis
cell growth
regulation of neuron projection development
cell−cell signaling by wnt
Wnt signaling pathway
synapse organization
gland development
regulation of nervous system development
negative regulation of phosphate metabolic process
negative regulation of phosphorus metabolic process
cell junction assembly
forebrain development
proteasome−mediated ubiquitin−dependent protein catabolic process
urogenital system development
regulation of neurogenesis
transmembrane receptor protein serine/threonine kinase signaling pathway
Ras protein signal transduction
positive regulation of cell projection organization
protein localization to cell periphery
renal system development
macroautophagy
regulation of small GTPase mediated signal transduction
telencephalon development
dendrite development
developmental growth involved in morphogenesis
regulation of axonogenesis
GeneRatio
Count
p.adjust

FIG. 5

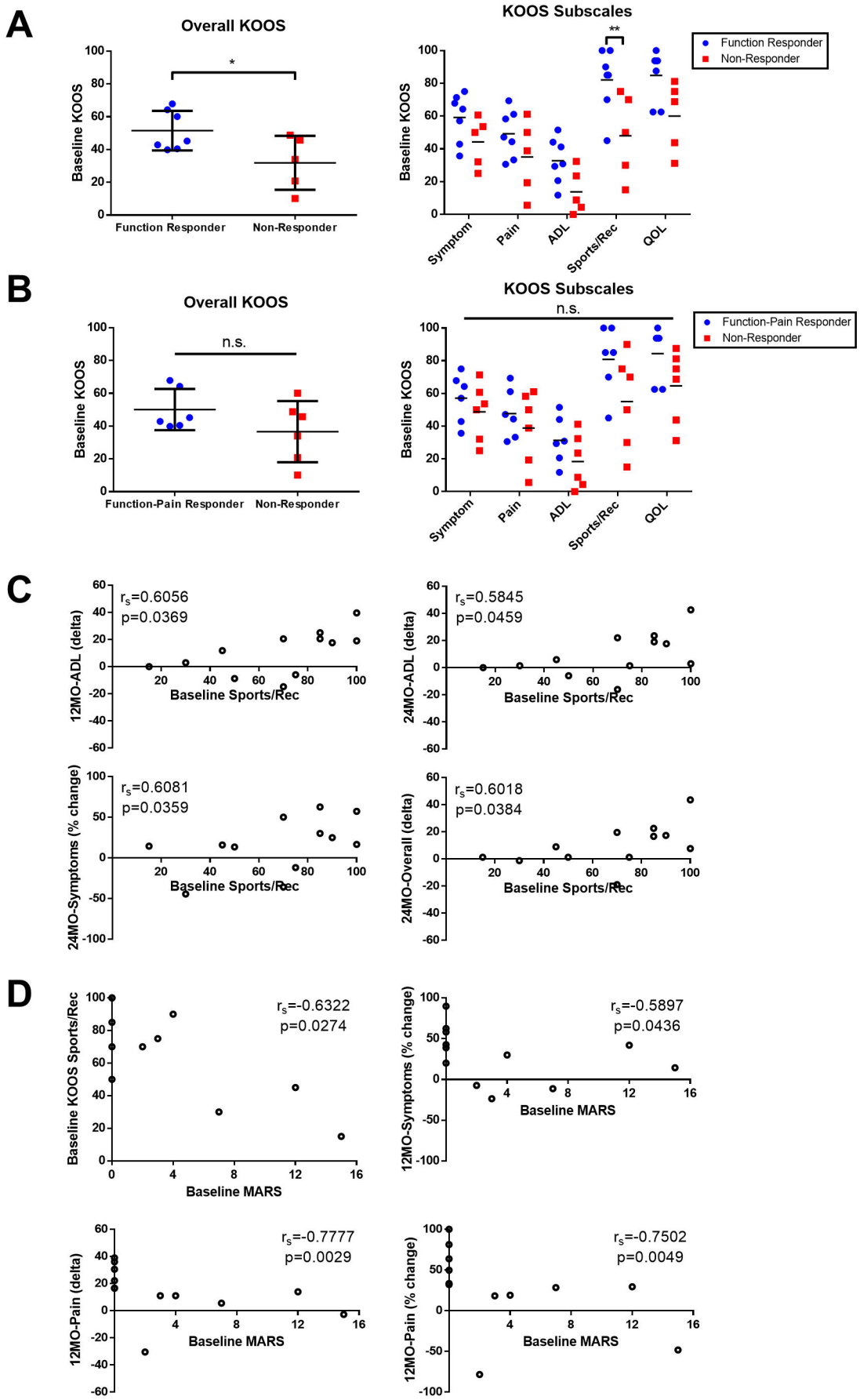
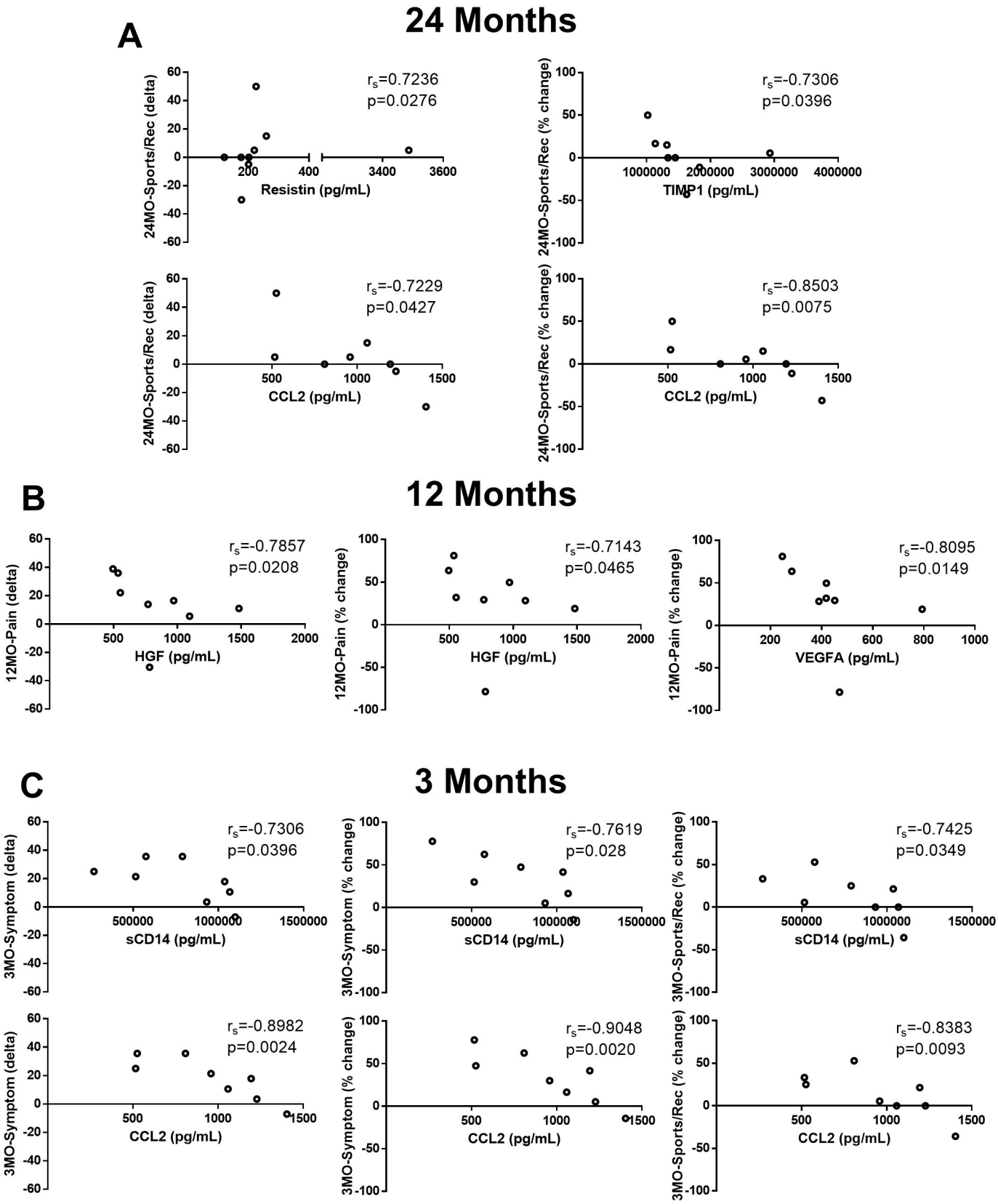

A
Overall KOOS
Baseline KOOS
Function Responder
Non-Responder
KOOS Subscales
Function Responder
Non-Responder
Symptom
Pain
ADL
Sports/Rec
QOL
B
Overall KOOS
n.s.
Function-Pain Responder
Non-Responder
KOOS Subscales
n.s.
Function-Pain Responder
Non-Responder
C
r_s=0.6056
p=0.0369
12MO-ADL (delta)
Baseline Sports/Rec
r_s=0.5845
p=0.0459
24MO-ADL (delta)
r_s=0.6081
p=0.0359
24MO-Symptoms (% change)
r_s=0.6018
p=0.0384
24MO-Overall (delta)
D
r_s=-0.6322
p=0.0274
Baseline KOOS Sports/Rec
Baseline MARS
r_s=-0.5897
p=0.0436
12MO-Symptoms (% change)
r_s=-0.7777
p=0.0029
12MO-Pain (delta)
r_s=-0.7502
p=0.0049
12MO-Pain (% change)

FIG. 6


A
24 Months
24MO-Sports/Rec (delta)
Resistin (pg/mL)
r_s=0.7236
p=0.0276
24MO-Sports/Rec (% change)
TIMP1 (pg/mL)
r_s=-0.7306
p=0.0396
CCL2 (pg/mL)
r_s=-0.7229
p=0.0427
r_s=-0.8503
p=0.0075
B
12 Months
12MO-Pain (delta)
12MO-Pain (% change)
HGF (pg/mL)
VEGFA (pg/mL)
r_s=-0.7857
p=0.0208
r_s=-0.7143
p=0.0465
r_s=-0.8095
p=0.0149
C
3 Months
3MO-Symptom (delta)
3MO-Symptom (% change)
3MO-Sports/Rec (% change)
sCD14 (pg/mL)
CCL2 (pg/mL)
r_s=-0.7306
p=0.0396
r_s=-0.7619
p=0.028
r_s=-0.7425
p=0.0349
r_s=-0.8982
p=0.0024
r_s=-0.9048
p=0.0020
r_s=-0.8383
p=0.0093