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

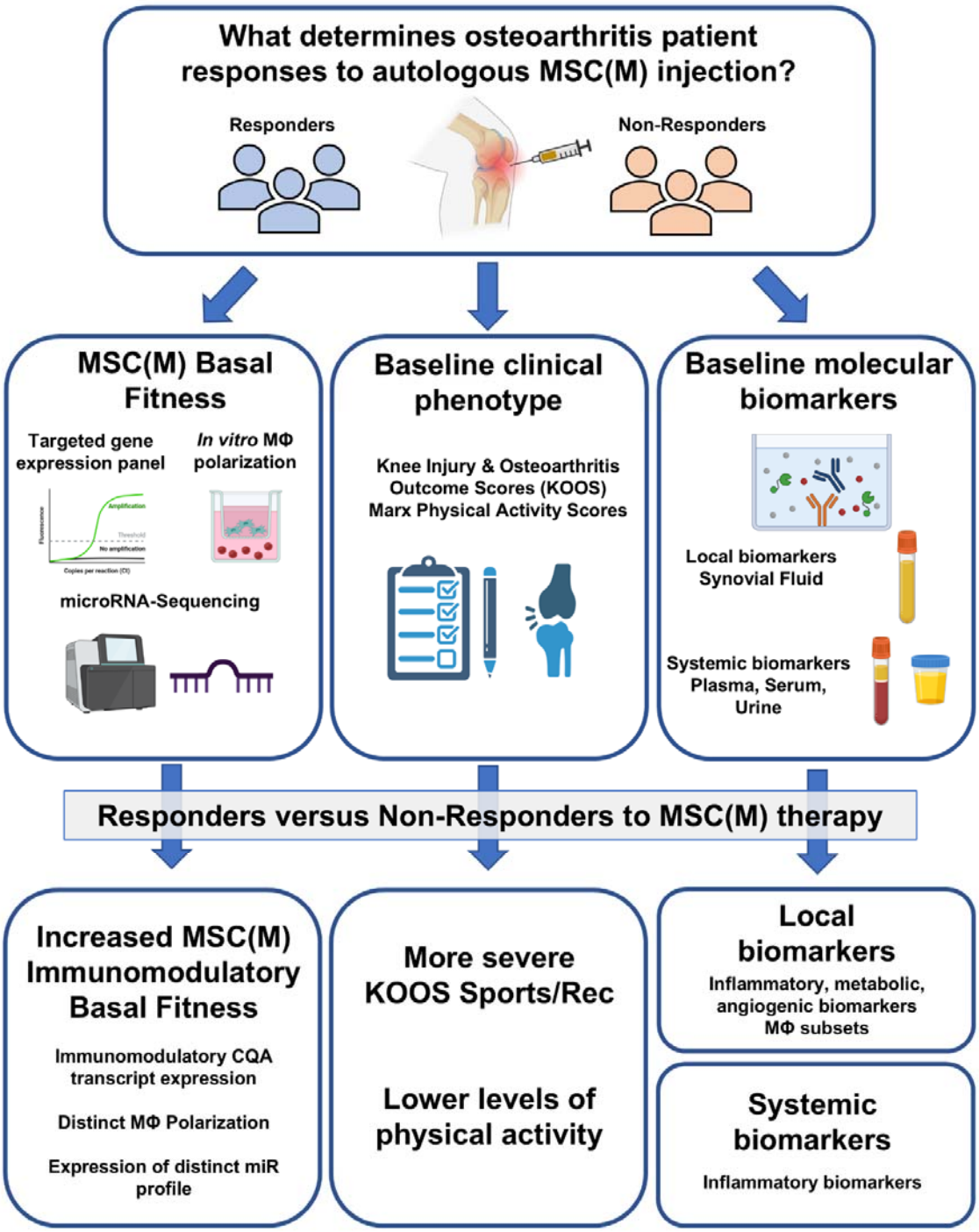
Objective: Despite recent approvals, mesenchymal stromal cells (MSCs) have shown mixed efficacy in advanced clinical trials of knee osteoarthritis (KOA). Analysis of contributing factors to the underlying heterogeneity in patient responsiveness is needed for successful clinical translation.

Methods: We provide a novel algorithm that correlates responder status to: i) baseline immunomodulatory attributes of bone marrow-derived MSC (MSC(M)) batches, and to ii) KOA baseline clinical features, local and systemic biomarkers. Using previous clinical trial with twelve batches of autologous MSC(M) as a test dataset, we demonstrate correlations with new longitudinal medium-and long-term clinical data.

Results: MSC(M) in KOA clinical responders have reduced expression of angiogenic genes, *THBS1*, *CXCL8*, and *ANGPT1* and higher expression of growth factor *CCN2* that correlates with 12, 24-month and 8–10-year improvements in patient outcomes. Responder MSC(M) have distinct microRNA signatures and significantly higher *in vitro* ability to modulate monocyte phenotypes. Clinical responders had greater OA baseline severity; reduced physical activity; lower local baseline levels of CCL2, VEGFA, HGF; higher local baseline of resistin. Leave-out-one sensitivity analysis confirmed findings, mitigating sample size concerns. Importantly, 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 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 and defining critical quality attributes to reliably identify potent MSC batches.

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 fluctuate temporally [2]. Culture-expanded mesenchymal stromal cells (MSCs) are being investigated for their multimodal mechanisms of action, including immunomodulatory functions [3,4]; however, there has been conflicting clinical efficacy reported including Mautner *et al*. who showed umbilical cord-derived MSCs were equivalent to corticosteroids in terms of improvements in pain or cartilage magnetic resonance imaging scores [5]. Contrastingly, other reports [6–9], meta-analyses [10,11], and randomized controlled trials [12,13] showed symptom and structural modification. Do MSCs work in KOA? Can we identify differences between responder vs. non-responders to deliver effective MSC treatments to KOA patients?

Heterogeneous responsiveness to MSC treatments is due to an interplay of both basal potency attributes 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]; well-defined critical quality attributes (CQAs) that are sensitive, quantitative and relevant to disease specific mechanism of action are thus needed [17–19].

Using reported [8], and new follow-up data from a phase I/IIa dose-escalation clinical trial, we conduct an in-depth analyses of donor MSC batches and baseline/follow-up KOA patient characterization to provide an integrated algorithm to analyze heterogenous KOA patient responsiveness to MSC treatments. Using a previously reported gene panel that correlated to *in vitro* functional activity [20], there is significant discrimination of responder vs. non-responders MSC(M) from marrow tissue. MicroRNAs, important mediators of MSC [21] further discriminate MSC(M) from responders vs. non-responders.

KOA is recognized not as a single disease but a constellation of clinical phenotypes [22,23] and divergent molecular endotypes [24]. Here we report both clinical phenotypes and molecular endotypes in KOA patients responsive to MSC(M) treatments.

MATERIALS AND METHODS

Clinical trial and patient data

KOA patients (Kellgren Lawrence (KL) grade 3-4) were recruited to a nonrandomized, open-label, dose-escalation, Health Canada-authorized phase I/IIa clinical trial as reported [8] (ClinicalTrials.gov Identifier: NCT02351011). Ethics approvals including for long-term follow up was granted (REB 14–7909, REB 20-5066, REB 24-5463) [8]. 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. Calculation of patient-reported outcome measures (PROMs), including the inverted KOOS and the Marx Activity Rating Scale (MARS), as well as determination of patient responder status are described in **Suppl. File 1**.

MSC(M) isolation and culture

Biobanked MSC(M) were retained in liquid nitrogen as individual batches by consent of KOA patients. MSC(M) were thawed in three batches at P3 or P4 and plated directly on 24-well plates for microRNA-seq and gene expression analyses. 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. 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. Details on MSC(M) gene expression analysis (primers listed in **Table S1**), *in vitro* monocyte polarization, and microRNA-sequencing are described in **Suppl. File 1**.

Biomarker measurements

Levels of curated biomarkers in serum (see **Table S2**) collected at baseline prior to MSC(M) injection were measured previously [8]. Updated analyses and experimental questions were applied to the dataset in the present work. Additional inflammatory biomarkers were measured in baseline biobanked patient serum and plasma (analytes specified in **Table S2**) Synovial fluid collection was feasible for only nine patients. Synovial fluid immune cell profiling was done only on five patients from whom sufficient synovial fluid volume could be obtained at baseline and follow-up [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 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 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 using 12-month patient-reported KOOS data [8]. We applied OMERACT-OARSI criteria [25] to determine responder status, with modifications as described in **Suppl. File 1**. Patients were classified as responders vs. non-responders. Within the responder category, we identified three sub-categories (**Table S3**) including: i) Function-Pain Responders (exceeding thresholds in <u>both</u> KOOS ADL and Pain scores); ii) Function Responders (exceeding thresholds in ADL scores), iii) Pain Responders (exceeding thresholds in Pain scores); non-Responders did not exceed thresholds in either ADL or Pain (**Table S3**). Of twelve patients, eight Responders and four Non-Responders met these criteria. While the sample size is limited, the binary criteria is useful in determining factors affecting patient clinical responses. Correlations with all KOOS subscale scores are included to supplement the analyses. There was no observed dose-dependency; responders existed across all three MSC(M) doses including the 1×10^6 (2/4), 10×10^6 (1/4), and 50×10^6 (3/4) (**Table S3**). We report on Function Responders; other Responder category data are included in the supplementary files.

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

Patients categorized at 12 months as Function Responders displayed significantly improved KOOS scores in all subscales at 12- and 24-months follow-up, relative to baseline (**Fig. 1A**), supportive of persistent effects of a single injection of autologous MSC(M). Patients categorized as Non-Responders similarly showed no significant changes at 12- and 24-months relative to baseline, indicating stable clinical phenotype. No significant differences were observed when comparing outcomes at 12 vs. 24-months for any KOOS subscale in both responders and non-responders. Similar results were obtained when patients were sub-categorized as Function-Pain Responders (**Fig. S1A**) or Pain Responders (**Fig. S1B**).

We further analyzed patient follow-up at 8-10 years after MSC(M) injection. 12-month Responders maintained significant improvement in multiple KOOS subscales at 8-10 years follow-up (**Fig. S2**). Specifically, 7/8 responder patients maintained their responder status at 24 months (**Fig. 1B, 1C**). Of these, 5/7 maintained Responder status at 8-10 years, and one patient (underwent knee arthroplasty) did not provide long-term KOOS data. 2/8 Responder patients received no additional interventions; 2/8 received viscosupplementation or platelet-rich plasma; 3/8 patients underwent knee arthroplasty surgery.

4/4 12-month Non-Responder patients maintained status at 24-months, and one patient was lost to follow-up at the 8–10-years (**Fig. 1B, 1C**). 3/4 patients maintained Non-Responder status at 8-10 years, with 1/4 patients reporting no additional interventions and 2/4 patients reporting viscosupplementation.

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 gene panel that correlated with *in vitro* immunomodulatory functionality [20]. Pro-inflammatory licensing conditions were used as a common method to measure immunomodulatory genes in MSC(M) [26].

Principal Component (PC) analysis revealed distinct clustering between Function Responder vs. Non-Responder MSC(M) donor batches along the PC1 axis (29.3% of total variance), with higher expression of *CCN2* and lower expression of angiogenic markers *THBS1*, *CXCL8*, and *ANGPT1* contributing strongly to higher PC1 scores (**Fig. 2A**, **Fig. S3A**). PC1 scores were significantly lower for all categories of Responders vs. Non-Responders (**Fig. 2B**, **Fig. S3B**), confirming the discriminatory power of this curated gene panel. To ensure that individual donor batches were not skewing results, outlier analysis was performed by calculating T^2 statistic, identifying three outlier data points (two Responder, one Non-Responder); however, the PCA returned similar results even with these data points removed; clear separation of responder/non-responder was observed across the expected eigenvectors (**Fig. S3C**).

Higher basal gene expression of the angiogenic gene, *ANGPT1* in 9/12 MSC(M) donor batches strongly (Spearman's rho >0.68) and significantly negatively correlated to improvements in KOOS sub-scales and Overall KOOS at 12 months, as well as to

improvements in KOOS ADL at 24-months (**Table S4**). By 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 sub-scales, and Overall KOOS at 12-months. PC1 scores, indexed as a composite score for basal immunomodulatory gene expression profile, showed robust negative correlations with improvements in KOOS sub-scales and Overall KOOS at both the 12- and 24-months, as well as KOOS QOL (12-month only) and Pain (24-month only) (**Fig. 2C**, **Table S4**). Remarkably, there was continued significant negative correlations between PC1 scores with KOOS ADL and Overall KOOS at 8-10 years (**Table S4**).

To evaluate the strength of these correlations, we performed a leave-out-one sensitivity analysis in which correlations to the PC1 score were measured after omitting one patient at a time, focusing on changes in ADL, Pain, and Sports/Rec as key KOOS subscales (**Table S5**). For all Spearman's correlations to 12-month outcomes, all leave-one-out correlations maintained significance. At 24 months, the correlation to percent change in KOOS ADL maintained significance, while the significant correlation to percent change in KOOS Pain was lost. PC1 correlation to KOOS ADL at 8-10 years showed a reduced Spearman's rho value that was no longer significant. Taken together, these findings validate the robustness of this gene panel [20] to discriminate 12-and 24-month clinical responsiveness to MSC(M).

In vitro modulation of monocyte phenotype by donor MSC(M) batches correlates to patient responder status

Functional *in vitro* monocyte polarization using mismatched human peripheral blood monocytes further assessed baseline MSC(M) potency (**Fig. 3A**). Discriminant analysis demonstrated distinct clustering and greater separation of monocyte phenotypic profiles between Responder vs. Non-Responder donor MSC(M) batches (**Fig. 3B**). Co-cultures of monocytes with 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 Non-Responder vs. Responder MSC(M) donor batches. Overall, Responder MSC(M) donor batches modulated *in vitro* monocyte polarization towards a distinct pro-resolving phenotype. Discriminant analysis showed reduced separation with Function-Pain Responder (**Fig. S4A**) or Pain Responder (**Fig. S4B**) donor MSC(M) batches as indicated by greater misclassified data and reduced Entropy R^2 values (**Table S6**).

Specific microRNAs distinguish responder donor MSC(M) batches

To probe unbiased baseline differences in MSC(M) donor batches, microRNA-Seq was performed. PCA demonstrated clustering by donor batch and responder group rather than by synovial fluid stimulation (**Fig. 4A**). Thirty-three significant, differentially expressed microRNAs were identified between Responder vs. Non-Responder MSC(M) donor batches after correction for multiple comparisons; 11 microRNAs were upregulated, and 22 microRNAs were downregulated (**Fig. 4B**, **Table S7**). 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 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 in **Suppl. File 2**). Predicted gene targets did not include genes within the curated panel. However, correlation analysis of the top 10 differentially expressed microRNAs with the curated gene panel revealed statistical significant ($p<0.05$; hsa-miR-377-3p vs *ACTA2*; hsa-miR-1307-3p vs *THBS1*; hsa-miR-4775 vs *THBS1*) and trending ($p<0.1$; hsa-miR-338-5p vs *CCN2*; hsa-miR-483-5p vs *THBS1*; hsa-miR-4775 vs *CXCL8*; hsa-miR-210-3p vs *TNFAIP6* correlations (**Table S8**), suggesting indirect modulation of select genes by differentially expressed microRNAs. The microRNA-sequencing analysis provides further characterization and potential mechanistic insights into MSC(M) therapeutic effects in KOA patients.

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**). Function Responders displayed significantly elevated Overall KOOS and KOOS Sports/Rec at baseline compared to Non-Responders, supportive of more severe baseline clinical presentations in directing MSC responsiveness (**Fig. 5A**). Other responder categories however, displayed no significant differences (**Fig. S5A and S5B**).

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 S9**). The

baseline Sports/Rec scores positively and significantly correlated with improvements in: i) KOOS ADL at 12- and 24-months; in ii) KOOS Symptoms at 24-months; and iii) Overall KOOS at 24-months (**Fig. 5B**).

KOOS Sports/Rec subscale measures difficulty in knee function activities [27]; hence, we probed the relationship between the KOOS Sports/Rec score and physical activity using the MARS scores [28]. Correlation analyses demonstrated that higher baseline MARS scores (i.e., frequent physical activity) were significantly associated with lower (i.e., less severe) baseline KOOS Sports/Rec scores (**Fig. 5C**). Interestingly, a higher MARS score significantly correlated to worsening in i) KOOS Symptoms and ii) KOOS Pain at 12 months (**Fig. 5C**, **Table S10**). No significant correlations were observed for the 24-month timepoint, but baseline MARS score continued to have significant negative correlation with changes in KOOS Symptoms at 8-10 years follow-up (**Table S9, S10**).

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

The levels of baseline inflammatory, angiogenic, metabolic and cartilage extracellular matrix-associated biomarkers in synovial fluid were previously reported [8]; here we analyzed correlations with 12-, 24-month, and 8–10-year KOOS improvements (**Tables S11, S12**). There was significant **positive** correlation for resistin with greater improvements (delta values) in KOOS Sports/Rec scores at 24-months (r_s of 0.72; **Fig. 6A**), while all other significant correlations for inflammatory, chemotactic, and angiogenic factors were **negatively** correlated with follow-up improvements. Specifically, lower levels of TIMP1 significantly negatively correlated to percentage changes in KOOS Sports/Rec scores at 24-months (r_s of -0.073). CCL2 significantly negatively correlated with changes in KOOS Sports/Rec scores at 24-months (r_s of -0.72, -0.85). Significant negative correlations were also observed between changes in KOOS Pain at 12-months with the levels of the pleiotropic HGF (r_s of -0.79, -0.71), as well as 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 (**Fig. 6C**, **Tables S13, S14**), which was the earliest timepoint when patients displayed improvements [8]. A covariance matrix for local biomarkers showing significant correlations is reported in **Table S15**. Briefly, TIMP1, resistin, HGF and VEGFA covary, while CCL2 and sCD14 covary, indicative of multiple pathways that influence MSC(M)-mediated clinical

responsiveness. There were no significant correlations between synovial fluid biomarkers and changes in KOOS at 8-10 years follow-up (**Table S11, S12**). There were no significant correlations between synovial fluid biomarkers and changes in KOOS at 8-10 years follow-up (**Table S11, S12**).

Leave-out-one sensitivity analysis showed significant average correlations for baseline HGF with 12-month KOOS Pain (delta only) and for VEGF with KOOS Pain (percent change only; **Table S16**). At 24-months, the average correlations between resistin and KOOS Sports/Rec (delta) and between CCL2 and KOOS Sports/Rec (percent change) maintained significance.

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

We analyzed synovial fluid immune cell subpopulations at baseline using previous data [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. S6A**) as well as $CD3^{+}CD4^{+}CD69^{+}$ and $CD3^{+}CD4^{+}CD25^{+}$ T helper cells (**Fig. S6B**) as an important immunophenotype; higher baseline frequencies of Mϕs and activated T cells are present in non-responders, although not significant given the limited sampling.

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

Systemic baseline inflammatory biomarkers were measured in the present study (sCD163; CRP; S100A8/A9, TNFα; IL-6) alongside previous data on systemic biomarkers of cartilage breakdown [8]) (**Table S17, S18, S19**). Higher levels of baseline plasma TNFα significantly positively correlated to percentage changes in KOOS Pain and QOL at 6-months (**Fig. S7A**, **Table S19**), while alarmin S100A8/A9 significantly negatively correlated to changes in KOOS QOL, and Sports/Rec at 3-months (**Fig. S7B**, **Table S19**). Systemic levels of biomarkers for cartilage breakdown showed no significant correlations to changes in KOOS at any time points. Systemic biomarkers thus showed limited sensitivity and no discernible profile in terms of correlations to short-term PROM improvements.

DISCUSSION

Statistically powered clinical trials using MSCs have reported conflicting results in KOA patients [5,12]. To address underlying factors that dictate heterogeneous 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 short-, medium- and long-term, 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 prospective KOA clinical trials (and retrospectively on existing datasets) to i) screen potent donor/batches of MSCs; and 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 a 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] with 12 manufactured, patient-specific lots of MSC(M), which was data-rich with multiple baseline biomarkers, and clinical readouts and offered an excellent test dataset to study MSC(M) batch variations. Leave-out-one sensitivity analysis confirmed the robustness of correlations with a basal MSC(M) gene panel and patient baseline synovial fluid biomarkers mitigating concerns regarding the robustness of the correlations being skewed by relatively smaller sample size.

We provide longitudinal 12-, 24-month and 8–10-year follow-up 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, and significantly sustained responder and non-responder status 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, nor can we rule out the effects of additional interventions that may have contributed to the long-term outcome measures beyond the study endpoint [5,12].

Notably, our analysis demonstrating patients with severe KOA were more responsive to MSC(M) treatment aligns with findings in acute graft-versus-host disease [29], myocardial infarction [30] and ulcerative colitis [31], where more severe disease patients responded better to MSC treatments. Function Responders showed correlations to MSC(M) donor potency and a distinct baseline clinical phenotype (especially KOOS Sports/Rec scores). Although immunomodulatory therapies may be expected to induce greater analgesic effects [32], MSCs act through multimodal mechanisms with pain and functional improvements 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 KOOS ADL and Sports/Rec subscales [27]. Patients with higher baseline MARS scores also had lesser improvements, suggesting that patients who may have persisted through pain in physical activity may have exacerbated disease progression [31].

We focused on basal immunomodulatory properties of MSC(M), especially their ability to modulate monocytes, given their importance as a clinically relevant mechanistic target in KOA [4,7,8,33]. Our previously identified immunomodulatory gene panel for MSC(AT) [20] successfully distinguished MSC(M) donors classified under all categories of Responders vs. 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- and 24-months, assuring the individual donors were not skewing the reported correlations. 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) [34,35]. While the transcriptome of MSC(AT) and MSC(M) are distinct [36], 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. [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 [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 basal fitness of the cells [37,38], as well as baseline patient disease status [39].

MSC(M) derived from Responders modulated $CD14^+$ monocytes to more pro-resolving subtypes *in vitro* [8,40]. Although there was preferential upregulation of pro-resolving $CD14^+$ monocyte markers, we also observed upregulation of pro-inflammatory markers. Reliance on dichotomized gene expression markers is not indicative of the plurality of monocyte functionality [40]; rather induction of mixed monocyte phenotypes by MSCs is important for achieving therapeutic effects [41]. We used LPS after co-culture with MSC(M) to elicit stronger gene expression readouts from $CD14^+$ monocytes; LPS, present in synovial fluid of KOA patients, correlates with increased disease severity [42], justifying its inclusion in the assay.

MSC(M) from Function Responders had a significantly different microRNA signature compared to Non-Responders, which correlated with select genes in the curated gene panel. 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) in KOA [43]. Surprisingly, our analysis identified enrichment of several neuronal processes; however, given the role of innervation and neuronal signaling in joint pain in KOA [44], these microRNAs may be implicated in mediating analgesic effects of MSC(M). The functionality of these microRNAs requires further study, but these differentially expressed microRNAs may also serve as additional CQAs for identifying basally fit MSC(M).

Responders 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 [45], lower levels of HGF, TIMP1, VEGFA, CCL2, and sCD14 indicate lower levels of active local inflammation, immune cells and angiogenesis [46–49]. Low levels of sCD14 have been previously correlated to lower levels of activated neutrophils and macrophages in KOA [47] and to lower levels of $CD14^+CD16^+$ intermediate synovial fluid MΦs [33]. We

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 [31,50]. However, synovial fluid collection and local biomarker measurement was possible for only a subset of patients who likely had increased inflammation [51,52], suggestive of a selection bias for patients with active synovitis. Thus, KOA patients with an inflammatory phenotype and active disease presentation may be more responsive to MSC(M) treatments

There were only short-term significant, albeit mixed correlations between systemic biomarkers such as TNFα (plasma) and S100A8/A9 (serum) which positively and negatively correlated to PROM improvements, respectively. Interestingly, a similar mixed signature of baseline systemic inflammatory biomarkers has been reported in responders versus non-responders to allogeneic Wharton's Jelly MSCs in rheumatoid arthritis patients [61]. Baseline serum CRP levels, an inflammatory biomarker in multiple inflammatory conditions [53] failed to correlate with improvements in KOOS at all time points as did cartilage breakdown biomarkers, previously shown to correlate with worsening KOA pain [54]. Overall, our analyses suggest that local biomarkers provide improved sensitivity.

Limitations of this study include small sample size, which we rigorously addressed with multiple sensitivity analyses. The absence of a control group is also a limitation, although effects were measured at multiple timepoints including 12-, 24-months and 8-10 years. KOA is a progressive condition [55], with approximately 30% of patients displaying worsening outcomes over two years leading up to joint surgery [56]. Although we cannot rule out placebo effects, we saw less than 30% of patients reported worsening outcomes, potentially speaking to a treatment effect. Indeed, analysis of Non-Responders using the rigorous OMERACT-OARSI criteria [25] showed that even patients within this category did not show any significant worsening in PROMs over 24-month and 8-10 years of follow-ups.

CONCLUSIONS

We demonstrated that donor 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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Figure Legends

Fig. 1. Osteoarthritis patient responders demonstrate improvements in KOOS subscales that are maintained out to 24 months follow-up post-injection. A) Patients categorized as Function Responders 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. B) Flow chart summary of patient responder status at 12-months, 24-months, and 8-10-years follow-up. C) Summary of OARSI-OMERACT Responder classification in 2025 after 8-10 years of follow-up. Responder status is indicated as Function-Pain (F-P), Function (F), or Pain (P) Responders. 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 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. 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) Summary of MΦ gene expression after co-culture with Function Responders MSC(M). 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.

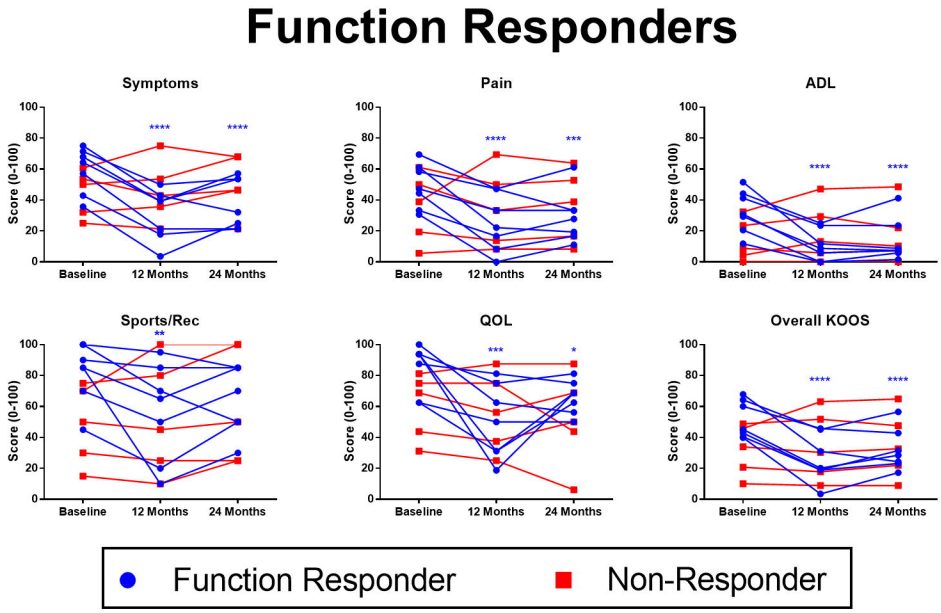
Fig. 5. More severe KOOS and lower MARS physical activity scores correlate to improved patient-reported outcomes at 12- and 24-months follow-up. A) Patients categorized according to Function Responders displayed significantly more severe overall KOOS and Sports/Rec scores at baseline relative to Non-Responders. B) 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. C) 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.

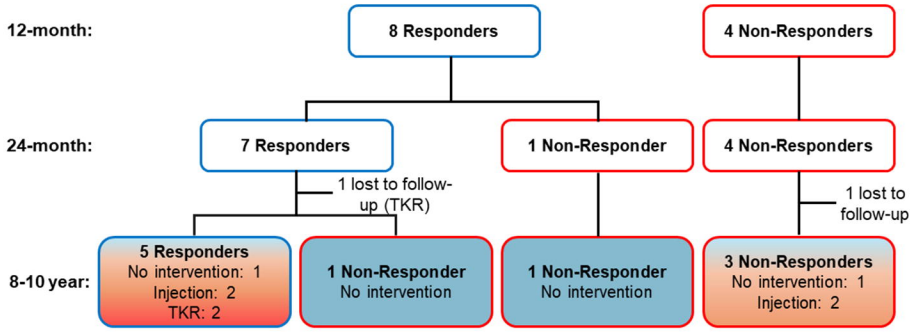
Sports/Rec: function in sport and recreation; MO: month; KOOS: knee injury and osteoarthritis outcome score; TIMP1: tissue inhibitor of metalloproteinases 1; CCL2: chemokine C-C motif ligand 2; HGF: hepatocyte growth factor; sCD14: soluble CD14; VEGFA: vascular endothelial growth factor A.

FIG. 1

A



B



C

					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	Responder (F-P)	Responder (F)	Responder (F-P)	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	Responder (F-P)	Responder (P)	N/A	-	-	-	-	-	TKR in 2025
6	Responder (P)	Pain Responder	Responder (P)	9 years, 0 months	11.11	22.22	-22.07	-501.59	Awaiting bilateral knee replacement
7	Responder (F-P)	Responder (F-P)	**Non-Responder**	8 years, 11 months	0.04	0.13	-1.46	-7.09	No intervention post injection
9	Responder (F-P)	Non-Responder	**Non-Responder**	8 years, 6 months	-2.82	-4.06	-19.14	-43.40	No intervention post injection
10	Responder (F)	Responder (F-P)	Responder (F-P)	8 years, 6 months	19.41	33.29	20.61	50.02	Viscosupplementation - no majory surgery
11	Responder (F-P)	Responder (F-P)	Responder (F-P)	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	Responder (F-P)	Responder (F-P)	Responder (F-P)	8 years, 2 months	49.99	81.82	39.74	77.17	TKR in 2025

FIG. 2

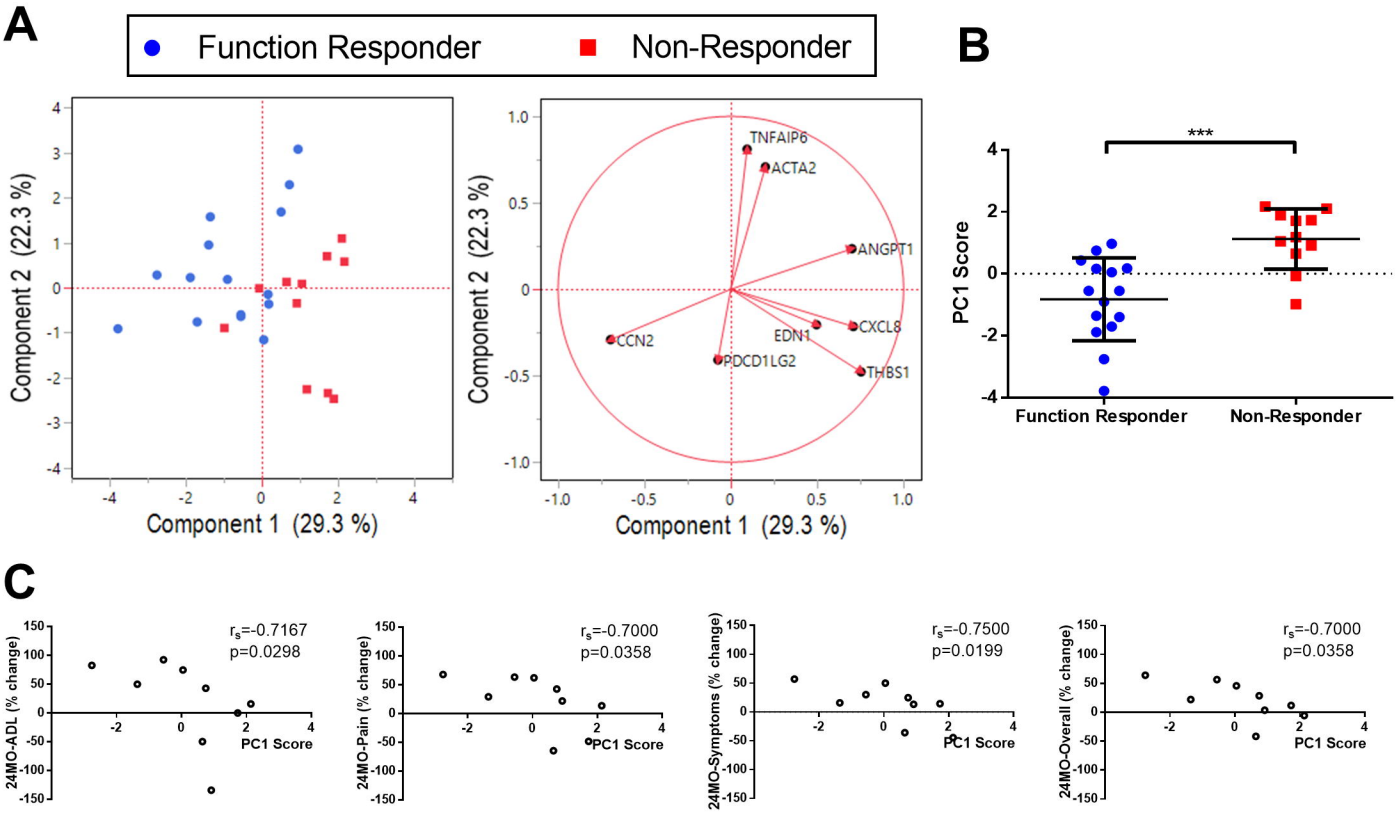

A
Function Responder
Non-Responder
Component 2 (22.3 %)
Component 1 (29.3 %)
TNFAIP6
ACTA2
ANGPT1
CXCL8
EDN1
CCN2
PDCD1LG2
THBS1
B
***
PC1 Score
Function Responder
Non-Responder
C
r_s=-0.7167
p=0.0298
24MO-ADL (% change)
r_s=-0.7000
p=0.0358
24MO-Pain (% change)
r_s=-0.7500
p=0.0199
24MO-Symptoms (%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

FIG. 3

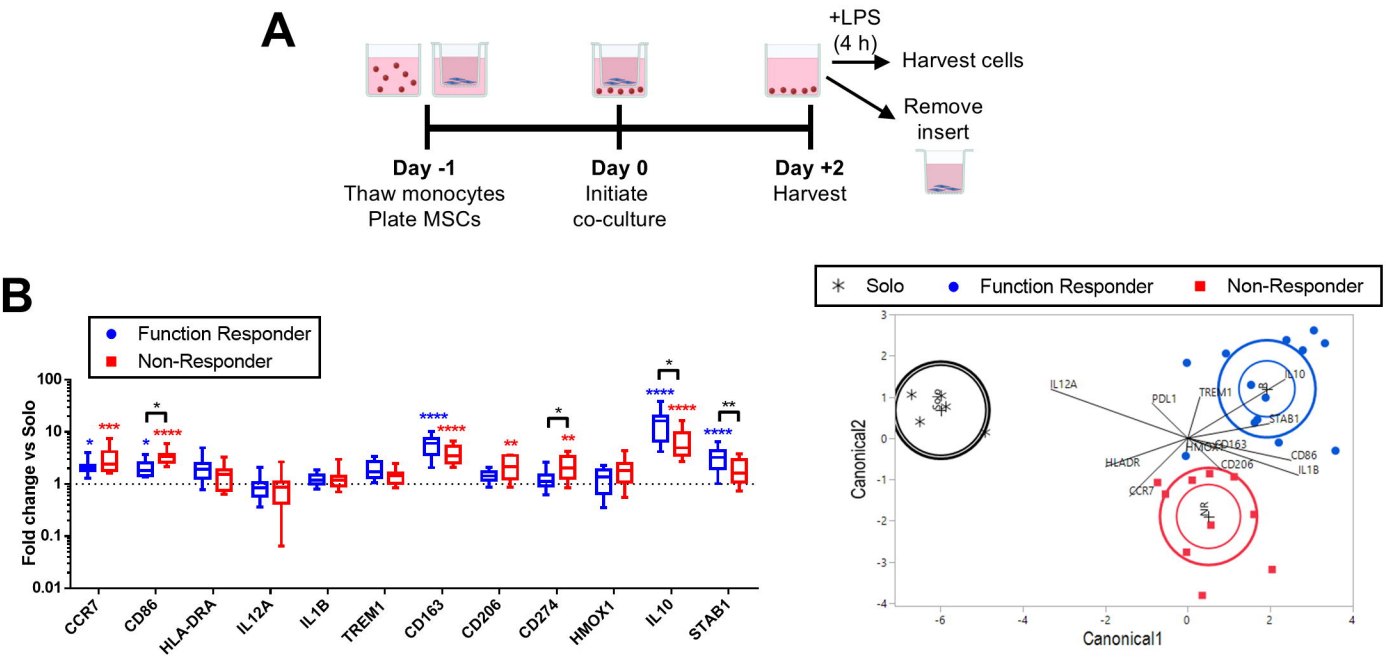

A
+LPS
(4 h)
Harvest cells
Remove
insert
Day -1
Thaw monocytes
Plate MSCs
Day 0
Initiate
co-culture
Day +2
Harvest
B
Function Responder
Non-Responder
Fold change vs Solo
100
10
1
0.1
0.01
CCR7
CD86
HLA-DRA
IL12A
IL1B
TREM1
CD163
CD206
CD274
HMOX1
IL10
STAB1
Solo
Function Responder
Non-Responder
Canonical2
Canonical1
IL12A
PDL1
TREM1
IL10
STAB1
HLADR
CD163
CD206
CD86
IL1B
CCR7

FIG. 4

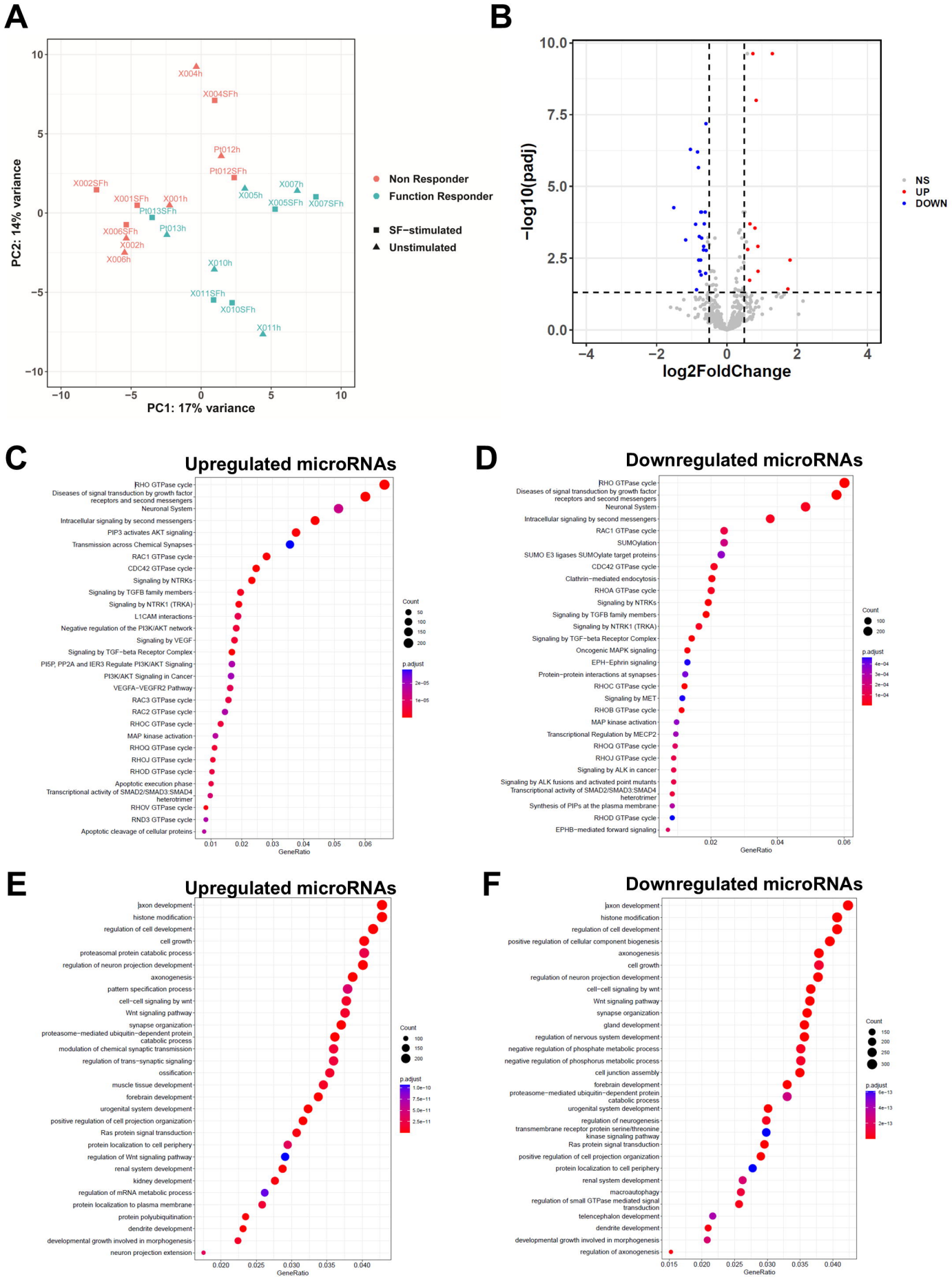

A
PC2: 14% variance
PC1: 17% variance
X004h
X004SFh
Pt012h
Pt012SFh
X002SFh
X001SFh
X001h
Pt013SFh
X006SFh
Pt013h
X002h
X006h
X005h
X007h
X005SFh
X007SFh
X010h
X011SFh
X010SFh
X011h
Non Responder
Function Responder
SF−stimulated
Unstimulated
B
−log10(padj)
log2FoldChange
NS
UP
DOWN
C
Upregulated microRNAs
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

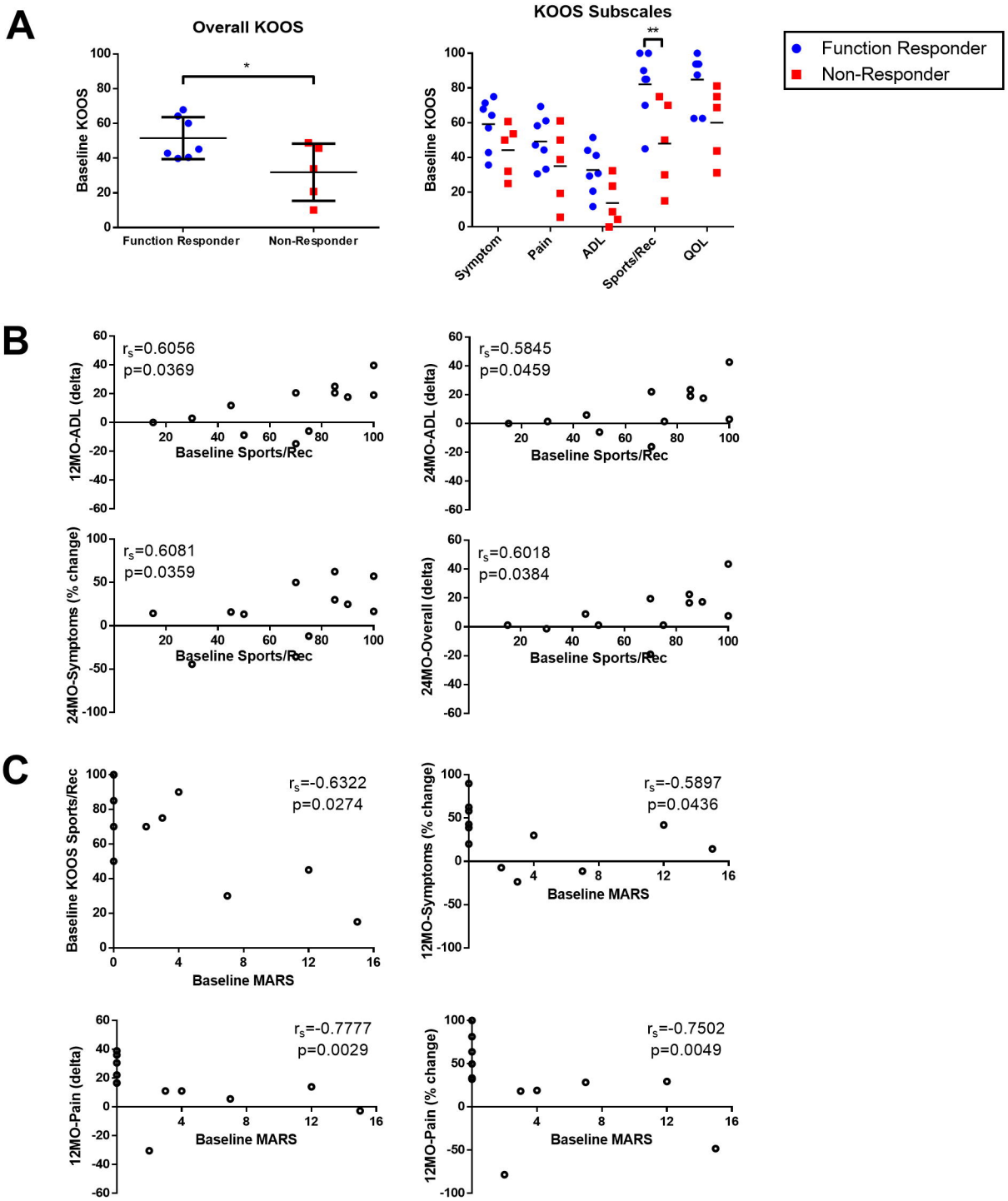
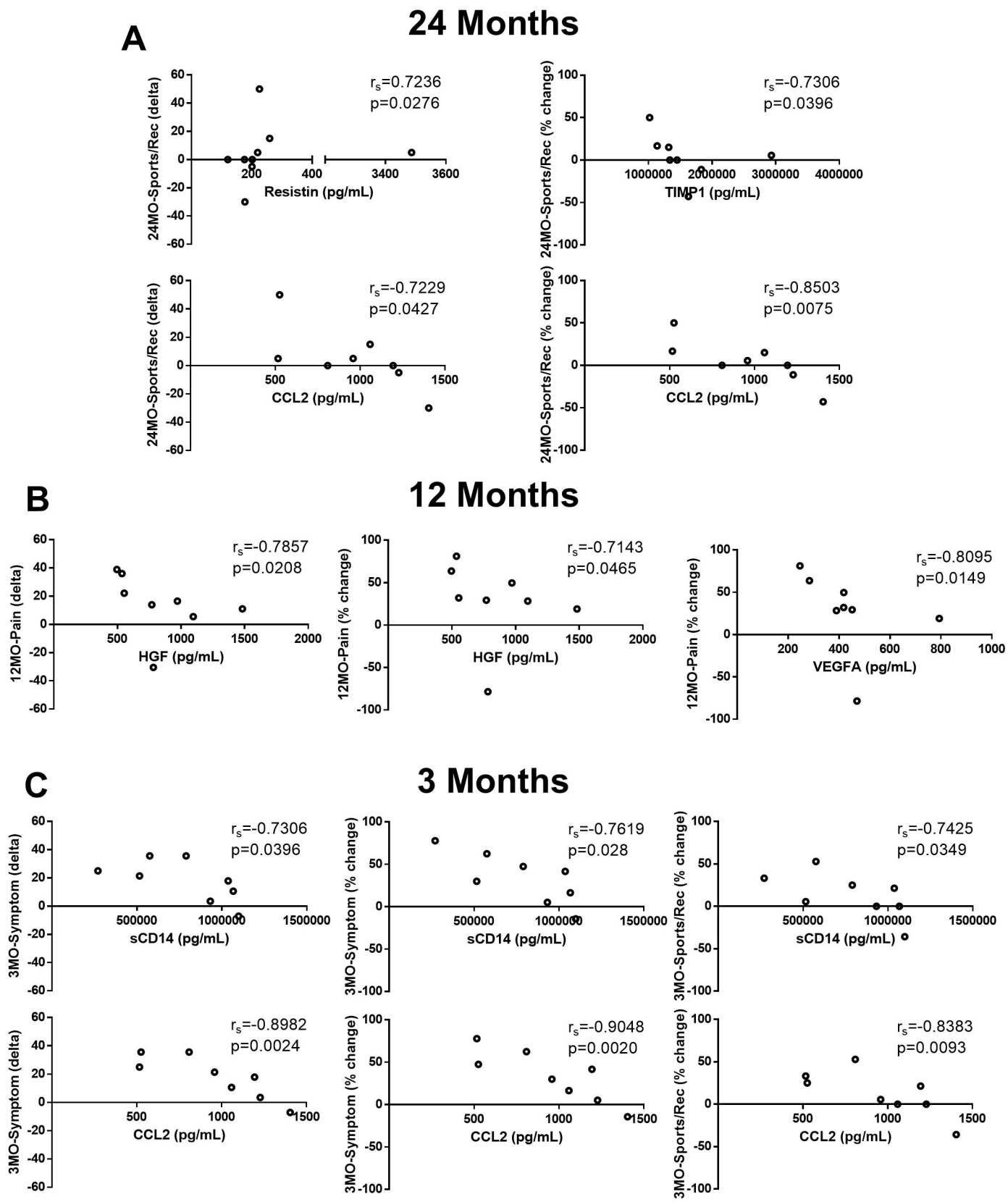

A
Overall KOOS
Baseline KOOS
Function Responder
Non-Responder
KOOS Subscales
Symptom
Pain
ADL
Sports/Rec
QOL
Function Responder
Non-Responder
B
r_s=0.6056
p=0.0369
12MO-ADL (delta)
Baseline Sports/Rec
r_s=0.5845
p=0.0459
24MO-ADL (delta)
r_s=0.6081
p=0.0359
24MO-Symptoms (% change)
r_s=0.6018
p=0.0384
24MO-Overall (delta)
C
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)
r_s=-0.7857
p=0.0208
r_s=-0.7143
p=0.0465
VEGFA (pg/mL)
r_s=-0.8095
p=0.0149
C
3 Months
3MO-Symptom (delta)
3MO-Symptom (% change)
3MO-Sports/Rec (% change)
sCD14 (pg/mL)
r_s=-0.7306
p=0.0396
r_s=-0.7619
p=0.028
r_s=-0.7425
p=0.0349
CCL2 (pg/mL)
r_s=-0.8982
p=0.0024
r_s=-0.9048
p=0.0020
r_s=-0.8383
p=0.0093