sagittal plane features are different at baseline in those that progress to total knee arthroplasty (TKA) compared to those that do not. However, no modelling using gait biomechanics has predicted progression to TKA (a clear endpoint that includes structure and symptom aspects of progression), and structural progression prediction models only included individual frontal plane features. This study determined: i) how well individual three-dimensional (3D) lower limb biomechanical gait features discriminated between those who progress to TKA and those that do not, ii) if a multivariate model improved discrimination ability, and iii) how well gait biomechanical features that best discriminated between groups predicted progression to TKA.

Methods: 54 knee OA patients underwent baseline gait analysis where 3D lower limb motion and ground reaction forces were recorded. 3D hip, knee, and ankle angles were expressed in the joint coordinate system. External moments were calculated using inverse dynamics and amplitude-normalized to body mass (Nm/kg). Waveform shape and magnitude features were extracted using Principal Component Analysis (PCA), and waveforms were scored based on how closely they matched an extracted pattern. PC scores were used in statistical testing. Knee adduction moment (KAM) peak (Nm/kg) and impulse (Nm/kg*s) were also calculated. 5-8 years later, 26 patients reported having TKA. Receiver operating characteristic (ROC) curve analysis determined discriminative abilities of individual gait variables. Stepwise discrimination analysis determined which multivariate combinations discriminated between groups. Discriminant function scores were calculated based on multivariate models, and used as input for additional ROC curve analyses to determine which multivariate model best discriminated between groups (highest area under ROC curve). Logistic regression analysis determined predictive ability of the multivariate model with the highest discrimination.

Results: There were no significant baseline differences in demographic, clinical and spatiotemporal gait characteristics, but 3D hip, knee, and ankle gait features significantly discriminated between TKA and no-TKA groups. KAM impulse resulted in the best univariate discrimination based on area under the ROC curve (AUC=0.79) with overall shape and magnitude of KAM (KAMPC1) having the second-highest AUC. Multivariate models had higher AUCs ranging from 0.80-0.85. In all multivariate models, overall KAM magnitude (impulse or KAMPC1) was the most dominant variable for progression to TKA. The multivariate model with the highest discrimination and correct classification ability (AUC=0.85, 74.1% correct classification rate) contained KAMPC1, difference between the early stance knee flexion and late stance knee extension moments (KFMPC2), and stance ankle dorsiflexion moment (AFMPC4). Higher overall KAM magnitude, decreased knee flexion to extension moment difference, and decreased stance dorsiflexion moment were associated with progression to TKA. This model had an odds ratio of 5.7.

Conclusions: Non-frontal plane moments were predictive of progression to TKA. Multivariate models were better able to discriminate between groups than univariate models, with the best model capturing overall magnitude of loading (KAMPC1), and inability to unload the knee (KFMPC2, AFMPC4). This suggests that higher overall cartilage loading along with sustained cartilage loading were predictive of progression to TKA. These findings are consistent with animal models showing that sustained loading can lead to cartilage degradation and upregulation of inflammatory chemicals linked to knee pain. Sustained cartilage loading has not previously been identified as a risk factor for progression, and can be a new target in the development and evaluation of conservative interventions.

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ESTABLISHMENT OF REFERENCE INTERVALS FOR OSTEOARTHRITIS RELATED BIOMARKERS – THE FNIH/OARSI OA BIOMARKERS CONSORTIUM

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Purpose: A Reference Interval (RI) is the central 95% range - or normal range - for endogenous analytes of a healthy person. Traditionally, reference ranges for biomarkers are established using commercially purchased "normals" including "normal" blood donors. Such "normals" are rarely adequate controls for Osteoarthritis (OA) studies as they are rarely if ever ascertained for OA status. The objective of this ancillary study was to establish reference intervals for the FNIH/OARSI panel of OA related biochemical biomarkers using biospecimens from stringently phenotyped age appropriate controls. We hypothesized that the reference interval ranges for these 'super controls' will facilitate future formal FDA qualification of the FNIH/OARSI OA-related biomarker panel for OA diagnosis and prognosis and for monitoring the efficacy of intervention.

Methods: African-American (AA) and Caucasian (C) participants were selected from the Johnston County Osteoarthritis Project (JoCo OA) population-based study of OA. From among 1518 participants with biospecimens and up to 15-years of follow-up radiographic data, and 412 with biospecimens and radiographic data at one timepoint, participants were selected who had no radiographic evidence of knee OA (Kellgren-Lawrence [KL] 0 in each knee) or hip OA (KL 0 or 1 in each hip), and no knee or hip symptoms (pain, aching or stiffness on most days). Controls further had no radiographic hand OA (GOGO definition) or spine radiographic OA (disc narrowing = 0 and anterior osteophytes no greater than 1 at same level). This yielded a total of N=129 healthy 'super control' participants with minimal or no radiographic burden of disease, two-thirds of whom had not developed OA in 15 years of observation. Baseline serum and urine samples from these individuals were analysed in duplicate for the analytes listed in Table 1. The RIs were determined with the reference intervals establishment module of EP Evaluator (Data Innovations) using transformed parametric calculations (Box-Cox transformation), which transforms the data into a Gaussian model. The SD ratio (cut-off >1.5) of the EP Evaluator partitioning test was used to determine if separate reference intervals might be justified for population subclasses based on gender or ethnicity (SD ratio calculated as the larger SD divided by smaller SD of the two subgroups, male vs female and AA vs C). Spearman correlations were used to assess for a correlation between the biomarker concentrations and age and body mass index (BMI).

Results: The sample of 129 super controls consisted of 64% women, 34% African Americans, mean age 59 (SD 8.3, range 45-95) years, mean BMI 29 (SD 5.9) kg/m2. There were no out of range high values. Out of range low values were set at just below the lower limit of detection of the assay to compute the RI. The central 95% intervals for the 18 OA-related biomarkers are provided in Table 1. Based on SD ratio >1.5, separate reference intervals may be warranted on the basis of gender for sHA, sMMP-3, sCol2-1NO2, uCTXIb/Cr, uCTXII/Cr, uNTXI/Cr, uCol2-1NO2/Cr and on the basis of race for sCS846, sCOMP, sMMP3, sCTXI, sCPII, uCTXII/Cr, and uCol2-1No2/Cr.

Conclusions: The ability to diagnose and prognose OA with biomarkers is dependent on a clear understanding of normal reference intervals. For a highly prevalent and heterogeneous disorder such as OA, the lack of ascertainment of OA status leads to misclassification of controls. These well-phenotyped controls represent a similar age demographic to that of the Osteoarthritis Initiative-FNIH main study sample so they should provide an optimal reference control for the main study. To our knowledge, no comparable 'super control' sample has ever been characterized for such an extensive panel of OA-related biomarkers.

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LIPIDS AS MEDIATORS OF CHONDROGENESIS

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Purpose: We have previously demonstrated that cartilage matrix formation is improved in pellet co-cultures of human mesenchymal stromal/stem cells (MSCs) and human primary chondrocytes (hPCs) under normoxic culture conditions (21% O2). This co-culture effect is attributed to the MSC specific expression of FGF-1. Under hypoxic culture conditions (2.5% O2), we observed that there was a decrease in chondrogenic differentiation in co-cultures as compared to the normoxic culturing conditions. For clinical applications it is plausible that co-transplantation of MSCs and chondrocytes into the defect results in improved cartilage repair. Until now it remains unclear how FGF-1 expression is regulated under the reduced oxygen level normally present in the joint. It has been underlined that hypoxia (reduced oxygen availability) and expression of

Table 1
Reference Interval (RI) estimations, N=129 (serum concentrations reported in ng/ml except serum Col2-1 NO2 in nM); urine concentrations normalized to urinary Cr and reported in the units indicated)

Biomarker	Manufacturer	Mean (SD)	Central 95% Interval		Confidence Ratio	Significant Spearman Correlations ρ (p value]
			MIN	MAX		AGE/BMI
Serum COMP	Biovendor	1016.12 (650.19)	404	2070	0.15	None
Serum HA	Corgenix	55.08 (21.41)	34	83	0.13	ρ 0.28 (0.001) with age
Serum CPII	IBEX	1450.90 (401.08)	830	2362	0.14	None
Serum CS846	IBEX	112.90 (29.83)	74	164	0.13	None
Serum MMP-3	Invitrogen	16.86 (16.15)	3.32	50.94	0.20	None
Serum PIIANP	EMD Millipore	2879.90 (705.58)	1690	4431	0.13	ρ 0.27 (0.002) with age
Serum C1,2C	IBEX	407.13 (107.43)	228	646	0.13	None
Serum C2C	IBEX	278.23 (48.98)	196	383	0.13	None
Serum NTXI	Ostermark/ Alere	16.02 (5.06)	9	27	0.14	None
Serum CTXI	IDS	0.31 (0.22)	0.093	0.741	0.17	None
Serum Col2-1 NO2	Artialis	9.01 (4.42)	3.24	20.20	0.16	None
Urinary CTXII (ng/mmol Cr)	IDS	293.50 (521.23)	59.80	765.21	0.19	None
Urine NTXI (nmol BCE/mmol Cr)	Ostermark/ Alere	36.93 (21.37)	11	90	0.17	None
Urine (alpha) CTXI (μg/mmol Cr)	IDS	0.67 (0.58)	0.13	1.98	0.20	ρ -0.17 (0.052) with BMI
Urine (beta) CTXI (μg/mmol Cr)	IDS	2.54 (1.87)	0.49	8.06	0.20	None
Urine C2C (HUSA) (μg/mmol Cr)	IBEX	108.97 (62.09)	36	258	0.17	None
Urine C1,2C (ng/mmol Cr)	IBEX	0.017 (0.010)	0.0	0.04	0.14	None
Urine Col2-1 NO2 (nmol/mmol Cr)	Artialis	0.032 (0.022)	0.0087	0.0831	0.18	None

COMP=cartilage oligomeric matrix protein; HA=Hyaluronan; CPII=type II collagen carboxy propeptide cleaved following release of newly synthesized procollagen into matrix; CS846=chondroitin sulphate epitope of aggrecan; MMP-3= Matrix Metalloproteinase-3 also called stromelysin-1; PIIANP=N-propeptide encoded by exon 2 of type II collagen; C1,2C=C-terminal neoepitoe of ¾ piece generated by cleavage of types I and II collagen by collagenases; C2C=C-terminal neoepitoe of ¾ piece generated by cleavage of type II collagen by collagenases; NTXI=N-terminal telopeptide of type I collagen; CTXI=C-terminal telopeptide of type I collagen (also called CrossLaps), alpha CTXI contains the newly synthesized alpha form of aspartic acid, beta CTXI contains the age-related beta form of aspartic acid; Col2-1 NO2 ; CTXII=C-terminal telopeptide of type II collagen (also called CartiLaps); Cr=creatinine.
All urinary biomarkers are normalized to mmol/L urinary creatinine (Quidel).
Confidence Ratio is the average confidence interval width to the reference interval width; a value of 0.10 or less is desirable, values < 0.30 are considered acceptable.
Significant Spearman Correlations are defined as $p<0.05$.
BCE=bone collagen equivalents.

hypoxia-inducible factor 1 (HIF-1) are essential in maintaining cartilage homeostasis. Moreover, it has been shown that primary chondrocytes perform better under reduced oxygen levels. However, only limited research has been performed to understand how hypoxia in the joint environment might influence cellular performance. Differential expression of proteins, lipids and other components indicate the unique behaviour of cells under different environmental conditions. In this study we aimed to identify the mechanism leading to the loss of co-culture effect under hypoxic conditions.

Methods: We used Time-of-flight secondary ion mass spectrometry (TOF-SIMS) to study the presence of lipids in chondrocytes, MSCs and Chondrocyte/ MSC co-cultures under normoxia and hypoxia. We performed biochemical assays to capture overall lipid expression, quantification of total cholesterol and phospholipids. Alcian blue staining was used to visualize sulfated GAG deposition. qPCR was used to identify the modulation of FGF-1 and of chondrogenic markers such as SOX9 and COL2a1.

Results: TOF-SIMS data revealed that normoxia allows for a rich presence of lipids in co- and mono-culture of hMSCs and hPCs. The highest amount of lipids was found in chondrocytes. Under hypoxia the overall lipid content was significantly decreased. Oil Red O staining supported the TOF-SIMS data (figure 1). Principle component analysis (PCA) showed that specifically cholesterol and diacylglycerols were found to be more abundant under normoxia. Our biochemical analysis for cholesterol and phospholipids support these data. Under normoxic conditions GAG deposition was increased and the morphology of the matrix more resembled that of articular cartilage, when compared to hypoxic co-cultures. This was verified by a higher SOX9 mRNA expression in normoxic co-cultures.

We correlated the presence of lipids to the FGF-1 mRNA expression. Under normoxic conditions an increase in FGF-1 production was observed in co-cultures. This was accompanied by better chondrogenesis as compared to chondrogenesis in hypoxic co-cultures. When we blocked the availability of free cholesterol in cells under normoxic conditions, we found that the FGF-1 expression was negatively affected, indicating that cholesterol can directly regulate FGF-1 mRNA expression (figure 2).

Conclusions: We found that FGF-1 expression was dependent on rich lipid content in the cultures and that specifically cholesterol had a modulatory role. We conclude that higher cholesterol under normoxia might be responsible for the improved performance of co-cultured pellets by regulating FGF-1 gene expression. These findings facilitate a better understanding of mechanistic routes in in vitro co-cultures of MSCs and hPCs. Furthermore, our data indicate that a cell's lipid composition is dependent on oxygen exposure and that these changes in lipids either directly or indirectly influence chondrogenesis.

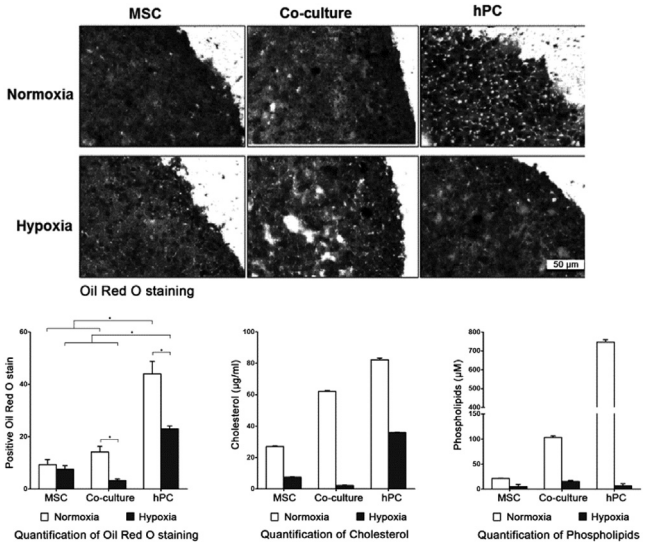

Figure 1. Top panel: Sections of pellets stained with Oil Red O for lipids. Bottom panel: Quantification of Oil Red O intensity (left), Quantification of Total cholesterol in pellets (middle), Quantification of Phospholipids (right).

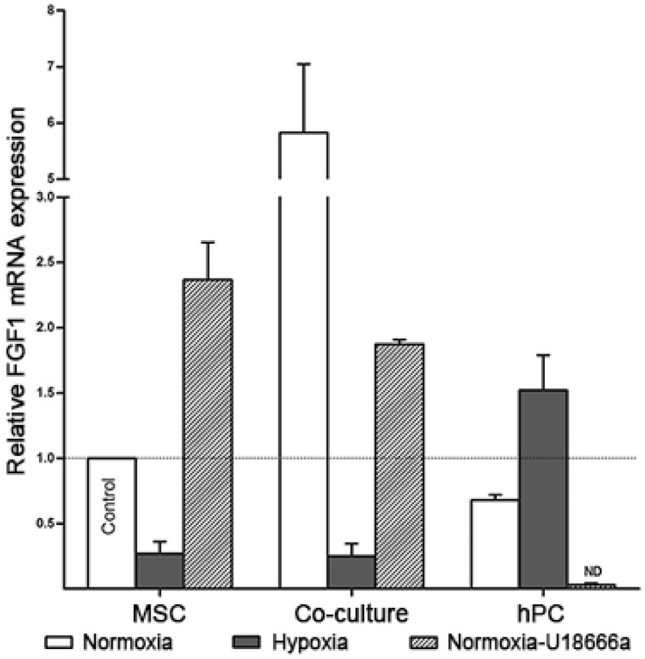

Figure 2. Relative expression of FGF1 mRNA under different culture conditions in co- and mono-cultures of hMSCs and hPCs.
Note: the first two authors contributed equally to this work.

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ARGS-AGGRECAN QUANTIFICATION IN SYNOVIAL FLUID, SERUM, PLASMA AND URINE - AN ASSAY VALIDATION

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Purpose: Aggrecanase cleavage at the 392Glu-393Ala bond in the interglobular domain of aggrecan, releasing N-terminal 393ARGS fragments into synovial fluid (SF), is an early key event in arthritis and joint injuries. We have shown that SF-ARGS is associated with radiographic progression of knee osteoarthritis (OA) as well as with progression of self-reported pain after meniscectomy. Our objective was to validate a modified ligand-binding assay for the detection of aggrecanase generated aggrecan fragments with the ARGS neoepitope in SF, blood and urine, and to verify the identity of aggrecan fragments found in blood.
Methods: A sandwich enzyme-linked immunosorbent assay (ELISA) on the Meso Scale Discovery (MSD) platform for detection of ARGS-aggrecan was validated, using a standard made by ADAMTS-4-digestion of recombinant human aggrecan (1220-PG, R&D Systems), an anti-aggrecan antibody (AHP0022, Invitrogen) for capture, and a monoclonal anti-ARGS (OA-1, GlaxoSmithKline) for detection. We analysed recovery to dilution and spiking of equimolar concentrations of standard in SF and serum samples. Matched samples of SF, serum, plasma, and urine were obtained from 36 subjects at different time points 0 to 5 years after an anterior cruciate ligament (ACL) tear, and analysed for ARGS-aggrecan content. Aggrecan was purified by caesium chloride density gradient centrifugation from serum and plasma pools and analysed by Western blot using the anti-ARGS antibody. We used Spearman's rank order correlation (rS) for assessment of correlation, based on Shapiro-Wilk test indicating skewed distributions of a majority of the variables.
Results: The limits of quantification for the ARGS-aggrecan assay was between 0.2 and 0.025 pmol ARGS/ml. Inside these limits recovery to dilution was, mean (range) 100% (83-117%), and to spiking 89% (77-110%). The sensitivity of the assay was improved 2-fold compared to when using a standard purified from human donors. The ARGS concentrations were highest in SF (mean, range; 3.02, 0.36 - 30.22 pmol/ml), 20 times lower in the blood samples (0.14, 0.055 - 0.28 pmol/ml serum and 0.13, 0.053 - 0.28 pmol/ml plasma), and 80 times lower in urine (0.036, <0.025 - 0.087 pmol/ml). Serum-ARGS and plasma-ARGS concentrations were similar, and correlated (Figure 1: rS = 0.773). SF concentrations correlated with serum and plasma concentrations (Figure 1: rS = 0.420 and 0.386, respectively). In purified serum and plasma samples, we identified 129-138 kDa aggrecan fragments containing the ARGS neoepitope.

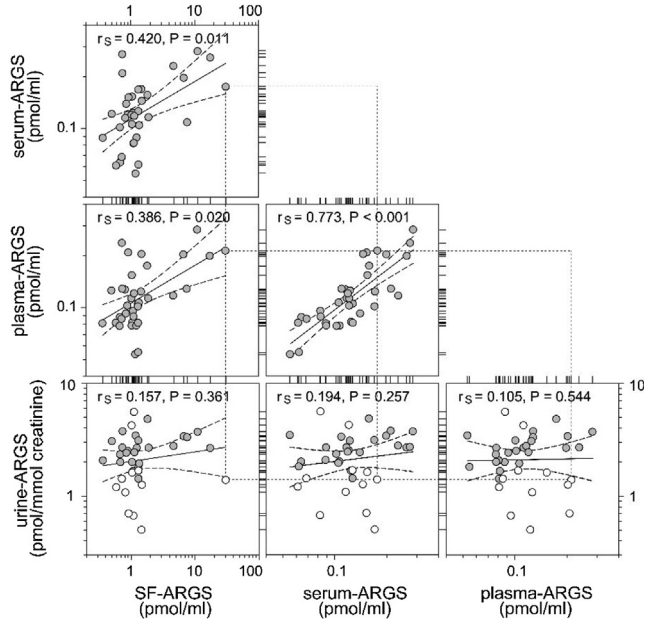

Figure 1. A rug-plot of bivariate scatters of ARGS-aggrecan concentrations in SF, serum, plasma and urine from 36 subjects at different times over 5 years after ACL injury. Concentrations in urine are given corrected for urinary creatinine. Filled circles are concentrations measured within the detection limits of the ARGS-aggrecan assay. Open circles are urinary ARGS concentration below the assay Lower Limit Of Quantification (LLOQ; 0.025 pmol/ml) that were imputed to half the LLOQ value. Linear regression lines (solid) with 95% confidence intervals (long dashed) are indicted in each scatter plot. In the rug-plot, each bivariate scatter plot shares information of one variate with the adjacent plot, illustrated by interconnecting rug fringes showing the marginal distribution of the shared variate. Horizontal and vertical lines (short dashed) interconnect ARGS concentrations in different bivariate combinations of fluids in one subject. All scales are logarithmic and reflect the range of ARGS concentrations in each fluid.

Conclusions: This novel ARGS-aggrecan assay is highly sensitive and suited for analysis of SF and blood samples. Both SF and blood contains ARGS-aggrecan, and ARGS concentrations in SF and blood are correlated.

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SPECIFIC PEPTIDE DISTRIBUTION IN HUMAN OSTEOARTHRITIC SYNOVIAL MEMBRANES REVEALS DIFFERENT GRADES OF TISSUE INFLAMMATION

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Purpose: The synovial membrane is an important source for biomarker discovery of many rheumatic pathologies. The heterogeneity of osteoarthritis (OA) affected tissues and the spatial distribution of proteins in the human synovial membrane is poorly studied. The specific protein and peptide profiles in different areas of the synovial tissue can reveal information about the underlying pathways that play a role in the OA pathology. The distribution of these specific protein/peptide profiles can be used for tissue/patient classification. Matrix assisted laser desorption ionization mass spectrometric imaging (MALDI-MSI) is a novel technique that can be employed to reveal the molecular signature of a region or area of interest in a biological tissue. In this work, we have studied the peptide profiles and their distributions in human normal and OA synovial membranes. We have described specific OA biomarkers commonly found in cartilage and synovial tissues by MALDI-MSI.