Inflammation : a link between metabolic syndrome and osteoarthritis?

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An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid

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Abstract

Soluble mediators, e.g. cytokines, chemokines and growth factors, are acknowledged as key players in the pathophysiology of osteoarthritis (OA) (1, 2). However, a wide-spectrum screening of such mediators in the joint environment is currently lacking. In this study, synovial fluid was collected from control donors and end-stage knee OA patients and analysed for 47 cytokines, chemokines and growth factors using several multiplex ELISAs. In addition, a principal component analysis (PCA) was performed to cluster the measured mediators. IL-6, IP-10, MDC, PDGF-AA and RANTES levels were found to be higher in OA compared to control synovial fluid ($p<0.001$). Leptin, IL-13, MIP-1β, sCD40L levels were higher and eotaxin and G-CSF levels were lower in OA synovial fluid than in control synovial fluid, albeit at borderline significance ($p<0.05$). Increased levels of inflammatory mediators and chemokines, such as MDC and IL-6, imply involvement of inflammatory processes in OA and might be associated with the influx of inflammatory cells in OA synovial tissue. Additionally, the PCA enabled identification of 6 different clusters, which explained 76% of the variance, and in this way could indicate different pathophysiological pathways in the joint. This dataset is valuable as a reference for future experiments to study pathophysiological pathways, and useful in more extensive profiling studies for OA.

Brief report

A control group of knee synovial fluid samples (n=16) were collected from *post mortem* donors within 24 hours after death. Control donors had no history of OA, other joint pathology and possessed macroscopic healthy cartilage. OA synovial fluid samples (n=18) were collected during total knee arthroplasty. All OA patients were diagnosed according to the ACR criteria for OA (3). Exclusion criteria were rheumatoid arthritis (RA) or infection. Synovial fluid samples were centrifuged at 3000 rpm for 3 minutes to spin down any cells or debris. The supernatant was stored at -80 °C until further analysis. The control synovial fluid samples were stored for 1 to 10 years and OA synovial fluid samples were stored for 1 to 3 years. None of the samples had ever been thawed before. Collection of the synovial fluid was done according to the Medical Ethical regulations of the University Medical Centre Utrecht and according to the guideline 'good use of redundant tissue for clinical research' constructed by the Dutch Federation of Medical Research Societies on collection of redundant tissue for research. As according to these guidelines, no information about the patients' characteristics could be obtained. Gender and age information was available for limited donors. Control donors had an average age of 39.6 ± 9.3 and consisted of 55% female. OA donors had an average age of 69.9 ± 7.9 and consisted of 64 % female. Due to the limited availability these data could not be linked to any of the outcomes.

Two hundred µl of each of the OA synovial fluid samples was pre-treated with 20 µl of hyaluronidase (Sigma, St, Louis, MO, USA; 10 mg/ml) for 15 min at 37 °C. Samples were spun down in a X-column (Corning, Amsterdam, Netherlands; Costar 8169). Finally, 150 µl of the synovial fluid sample was dissolved in 300 µl HPE-0.1375% Tween (Sanquin, Amsterdam, Netherlands). The pre-treated synovial fluid samples were used for all Multiplex ELISA assays mentioned below.

To determine a wide panel of soluble mediators the commercially available human inflammation 42-multiplex and the human adipokine 13-multiplex (Millipore, Bellirica, MA, USA) were used according to the manufacturer's protocol. Additionally, 12 different soluble mediators were measured with the Bio-Plex suspension system (Bio-Rad laboratories, Hercules CA, USA) as previously described (4). The levels of cytokines in the synovial fluid samples were expressed as pg/ml. All samples were

measured in the same plate and in duplo. Levels below the lower limit of quantification (LLOQ) were indicated as the value of the lowest point on the calibration curve divided by 2. The measured mediators are listed in table 1. Data are expressed as median ± interquartile range (IqR) as the data had a non-Gaussian distribution. IBM SPSS 20.0 software (IBM SPSS Inc. Chicago, IL, USA) was used for the statistical analysis.

For this study, a descriptive statistics approach was used for the interpretation of the data due to the limited sample size. A Mann Whitney-U test was used to assess differences between control and OA synovial fluid samples due to the predominantly non-Gaussian distribution of the data. As a descriptive statistic approach was used, values with a p-value less than 0.001 were considered significant. P values between 0.05 and 0.001 were considered borderline significant. No *post-hoc* correction for multiple testing was performed on the data, due to the descriptive approach.

PCA was performed to enable identification of clusters (i.e. components) of interrelated mediators within the complete dataset. Mediator levels were mean-centered to remove dependence on magnitude of levels. Control and OA patient data were combined in one single PCA analysis. Separate analysis of patients and controls was judged impossible due to the limited number of subjects. Only mediators with communalities > 0.3 were included. Loading factors were maximized using Direct oblimin rotation with Kaiser Normalisation. The optimum number of clusters was then decided based on the scree-plot and eigenvalues (> 1.0). Mediators were categorized per cluster when their loading scores were > 0.5. A Crohnbach's α was performed on each cluster to determine internal consistency.

This is one of the first studies in which such a comprehensive profile of soluble mediators was measured in synovial fluid from (end-stage) OA patients and control donors. With regard to previous studies, the pattern of the mediator levels was found to be compatible for OA donors. However, in these studies a small panel of mediators was measured and the inclusion of control donors was lacking (e.g. (5)). Table 1 provides an overview of the measured levels of each mediator (median ± IqR). The majority of the soluble mediators could be detected in the synovial fluid samples of both control and OA donors. Of the 47 measured mediators, 5 mediators were present at significantly different levels in OA compared to control synovial fluid. The levels of the chemokines MDC, RANTES and IP-10, the growth factor PDGF-AA and

the pro-inflammatory cytokine IL-6 were significantly higher in OA than in control synovial fluid ($p < 0.001$). In addition, levels of the adipokine leptin, the chemokine MIP-1β, and the pro-inflammatory cytokine sCD40L were higher and levels of the chemokine eotaxin and the growth factor G-CSF were lower in OA than in control synovial fluid, albeit all with borderline significance ($p < 0.05$).

Table 1 Overview of the measured mediator concentration (pg/ml) in control and osteoarthritic (OA) synovial fluid (SF).

	Healthy SF (Median ± IqR)	CV (%)	<LLOQ or 0 (n)	OA SF (Median ± IqR)	CV (%)	<LLOQ or 0 (n)	Detection limit	Mann-Whitney U (p-value)
EGF	4.8 ± 13.1	102.3	11	4.8 ± 0	189.3	14	5.3	0.65
Eotaxin	14.6 ± 39.6	96.0	6	0 ± 0	236.7	15	12.1	*0.02
FGF-2	37.2 ± 70.4	137.5	2	21.6 ± 231.4	134.1	4	16.0	0.67
Flt-3 ligand	135.9 ± 119.7	59.0	0	103.5 ± 76.4	53.1	0	6.1	0.23
Fractalkine	0 ± 0	387.7	14	0 ± 19	168.5	12	7.6	0.14
G-CSF	34.8 ± 151	105.9	1	16.9 ± 15	66.9	0	3.9	*0.03
GRO	59.2 ± 79.5	74.4	0	84.2 ± 96	81.6	0	11.4	0.38
IFNα2	7.2 ± 10.5	67.4	7	16.4 ± 46.8	89.3	7	27.2	0.09
IFNγ	40.7 ± 12.9	23.30	0	28.0 ± 21.0	136.74	2	2.14	0.1
IL-10	3.3 ± 6.3	127.6	6	2.1 ± 2.7	103.7	3	0.3	*0.04
IL-12 (p40)	4.8 ± 1.7	60.5	9	4.8 ± 11.9	137.7	10	12.4	0.30
IL-15	9.9 ± 6.0	37.1	0	12.8 ± 5.7	31.2	0	0.6	0.32
IL-1α	0 ± 1.8	217.8	9	0 ± 4.9	153.4	12	1.5	0.74
IL-1ra	0 ± 6.9	125.6	8	0 ± 0	288.4	14	2.3	0.14
IL-1ß	0 ± 1.5	205.4	11	4.8 ± 11.9	298.6	14	0.7	0.61
IL-3	0 ± 0	0.0	16	4.8 ± 0	179.3	16	9.8	0.18
IL-6	4.8 ± 0	196.9	13	135.8 ± 224.6	120.3	3	0.4	#0.001
IL-8	16.2 ± 43.5	92.2	0	30 ± 23.5	170.9	0	0.3	0.24
IP-10	302.1 ± 280.8	107.6	0	710.4 ± 597.1	93.1	0	1.3	#0.001
MCP-1	542.4 ± 839.2	102.1	0	824.8 ± 645.5	47.8	0	1.2	0.58
MCP-3	4.8 ± 36	123.5	10	4.8 ± 5	215.5	14	5.2	0.83
MDC	52.2 ± 38.4	43.9	0	189.5 ± 119.8	41.8	0	2.4	#0.001
MIP-1α	4.8 ± 0	26.7	16	4.8 ± 0	172.7	15	6.6	0.06
MIP-1ß	9.6 ± 24.0	83.7	5	21.8 ± 23.5	127.6	3	3.2	*0.04
PDGF-AA	0 ± 2.1	201.3	11	72.6 ± 116.9	72.8	0	0.3	#0.001
PDGF-AB/BB	43.2 ± 42.9	93.6	1	34.2 ± 69.7	137.8	0	12.2	0.44

RANTES	15.6 ± 28.1	152.7	0	408.2 ± 910.9	114.3	0	1.6	#0.001
sCD40L	0 ± 0	230.9	13	5.9 ± 45.1	144.6	7	5.2	*0.005
sIL-2ra	37.5 ± 76.8	90.8	1	63.0 ± 72.5	65.9	0	7.5	0.11
TGFα	0 ± 0.6	201.2	9	0 ± 0	401.5	17	1.4	0.4
HGF	2554.4 ± 3505.7	80.2	0	2303 ± 1695.2	38.12	0	1.6	0.32
Leptin	82.2 ± 1565.3	133.21	4	1637.5 ± 2414.1	125.33	0	27.4	*0.01
Resistin	2713.4 ± 1854.6	87.25	0	3824.8 ± 3550.7	72.71	0	4.5	0.12
Adiponectin	>250000	na	na	>250000	na	na	80.3	na
OSM	0 ± 0	447.83	16	0 ± 0	307.79	12	1.59	0.09

*Data are indicated as median ± Interquartile Range (IqR). The coefficient of variation (CV) in percentage and Lower Limited of Quantification (LLOQ) with the number of samples are given. Data are subjected to non-parametric statistical analysis Mann-Whitney U; *p<0.05 and # p<0.001. GM-CSF, IL-12(p70), IL-13, IL-17, IL-2, IL-4, IL-5, IL-7, IL-9, TNFα, TNFβ, VEGF and NGF could not be detected*

Table 2. Pattern matrix after Oblimin rotation with Kaiser Normalisation as obtained from PCA in the spectrum of synovial fluid mediators.

Action	Mediator	Cluster					
		1	2	3	4	5	6
Pro-inflammatory	IFNγ	0.988					
Pro-inflammatory	OSM	0.980					
Pro-inflammatory	IL-7	0.971					
Pro-inflammatory	IL-1β	0.947					
Anti-inflammatory	IL-1ra	0.851					
Chemokine	MCP-3	0.849					
Pro-inflammatory	IL-8	0.823					
Growth factor	TGFα	0.612					
Pro-inflammatory	IL-3		0.942				
Chemokine	MIP-1α		0.919				
Proliferation	EGF		0.888				
Adipokine	Leptin		0.871				
Proliferation	IL-12		0.702				
Chemokine	MCP-1			0.841			
Anti-inflammatory	IL-10			0.807			
Chemokine	Eotaxin			0.653			
Chemokine	G-CSF			0.622			
Chemokine	RANTES				-0.953		
Growth factor	PDGF-AB/BB				-0.935		
Growth factor	PDGF-AA				-0.914		
Chemokine	IP-10				-0.705		
Growth factor	sCD40L				-0.683		
Pro-inflammatory	IFNα				-0.589		
T-cell factor	Flt-3 ligand					-0.827	
Growth factor	HGF					-0.795	
T-cell factor	IL-15					-0.703	
T-cell factor	sIL-2ra					-0.565	
Adipokine	IL-6						0.922
Adipokine	Resistin						0.678
Eigenvalues		10.5	5.8	3.8	2.2	2.1	2.1
Variances explained (%)		30%	17%	11%	6%	6%	6%
Cronbach's alphas		0.835	0.059	0.114	0.615	0.070	0.115

Control and osteoarthritic samples are combined. Only loading factors > 0.5 are displayed.

The PCA was performed to gain insight into the associations between individual mediators that were assessed. The PCA showed communalities > 0.3, which as such, were included in the analysis. PCA enabled identification of 6 clusters of interrelated mediators among the spectrum of 47 mediators (Table 2). These clusters may reflect

important pathophysiological pathways in the joint. In the pattern matrix, the mediators IFNγ, OSM, IL-7, IL-1β, IL-8 and TGFα were clustered in the first cluster, and together explained 30.2 % of the total variance with an eigenvalue of 10.5. Cluster 2 included IL-3, MIP-1α, EGF, leptin and IL-12, (variance explained 16.8%, eigenvalue 5.9). Cluster 3, contained MCP-1, IL-10, eotaxin and G-CSF (variance explained 10.8%; eigenvalue 3.8). Cluster 4 contained RANTES, PDGF-AB/BB, PDGF-AA, sCD40L, IP-10, IFNα (variance explained 6.4%, eigenvalue 2.2). Cluster 5 included Flt-3 ligand, HGF, IL-15 and sIL-2ra (variance explained 6.1%, eigenvalue 2.1). Cluster 6 included the adipokines IL-6 and resistin (variance explained 6.0%, eigenvalue 2.1). In total, these 6 clusters explained more than 76% of the variance. The first two clusters mainly contained pro-inflammatory mediators and cluster 3 included predominantly chemokines. Cluster 4 contained predominantly growth factors and cluster 5 factors associated with T-cell proliferation and maturation. Finally, cluster 6 contained only adipokines. Mediators in all these 6 clusters are known to be part of important processes in the joint homeostasis. However, distinguishing between potential clusters was difficult, since these mediators are involved in adjacent and interrelated pathways.

Cytokines, chemokines, adipokines and growth factors play a major role in inflammatory diseases, such as OA, and are currently intensively studied. The adipokines are associated with obesity, which is in itself associated with low-grade systemic inflammation, one of the risk factors for the development and progression of OA (2, 6, 7). The different levels of IL-6, leptin and adiponectin between control and OA synovial fluid might indicate a role for certain adipokines in OA processes, which is in line with literature (7). However, these data were not corrected for BMI, which might influence the outcomes. Another group of mediators consisted of chemokines. The measured chemokines such as the related Chemokines-Chemokines (CC) mediators RANTES, MDC, MIP-1α and MIP-1β were higher in OA than in control synovial fluid. This group of chemokines share the same receptor complexes, e.g. CCR1, CCR2 and CCR5 (8) and their major function is to attract inflammatory cells, e.g. T-cells, macrophages and other inflammatory cells to sites of inflammation. Chemokines, such as RANTES, are also capable of activating (inflammatory) cells in the production of inflammatory mediators. As shown in previous studies, RANTES promotes the

production of IL-6 by synovial fibroblasts and enhances the inflammatory response in OA through the different CCR receptor in synovial tissue (9). Moreover, CCR receptors are present in chondrocytes and treatment of cartilage explants with RANTES was demonstrated to increase release of proteoglycans (10). The combination of multiple CC-chemokines in combination with IL-6 and leptin in the synovial fluid was not demonstrated earlier. Blocking these CC-chemokines or their receptors in OA might reduce IL-6 and leptin production and, consequently, the infiltration of inflammatory cells and the production of catabolic factors in the joint. Future research is necessary to elucidate this.

In none of the synovial fluid samples GM-CSF, IL-12(p70), IL-13, IL-17, IL-2, IL-4, IL-5, IL-7, IL-9, TNFβ, VEGF and NGF and TNFα, could be detected. IL-1 was only detected at very low levels. TNFα and IL-1 are pro-inflammatory cytokines, which are associated with cartilage degeneration, synovial inflammation and bone changes (11). Although in some *in vivo* animal models of OA, blocking IL-1 or TNFα gave promising results, this could not be validated in clinical studies (12). Our results likewise do not support a prominent role for IL-1 and TNFα in end-stage OA patients. Nonetheless, this does not exclude a role for IL-1 and TNFα in for example early OA, as OA has a heterogeneous character with multiple phenotypes.

For the interpretation of the data it should be mentioned that our results are based on a small sample size, due to donor availability, which were not paired on age, BMI and sex. Moreover, it should be taken into account that ex-vivo modifications cannot be excluded for the control donors as samples were taken after death. Also due to the small sample size, no further statistics were performed on the PCA to study differences between the clusters for OA and control synovial fluid samples. Therefore this pilot dataset should be regarded as a reference and more extensive profiling studies are necessary to confirm these data.

In summary, this is the first study measuring a wide panel of mediators in the synovial fluid of both control and end-stage OA donors. Increased levels of mediators such as MDC, IL-6 and RANTES once more confirm involvement of inflammatory processes and might be associated with the influx of inflammatory cells in OA synovial tissue. In addition, the PCA indicated 6 clusters, which reflect different processes in the joint. Due to the small samples size no hard conclusions can be drawn.

Nonetheless, this pilot dataset provides a valuable reference for future experiments to study pathophysiological pathways, and to be useful in more extensive profiling studies for OA.

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