Genes and mediators of inflammation and development in osteoarthritis

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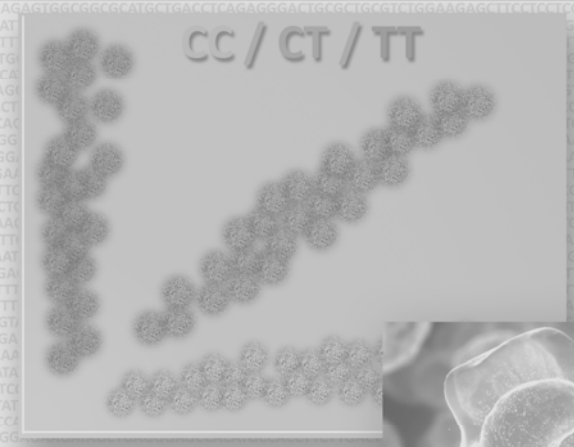

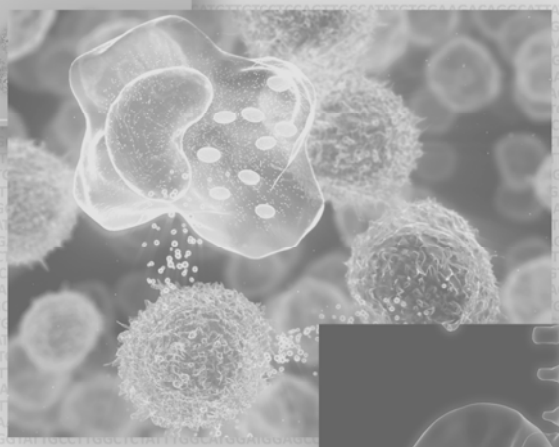

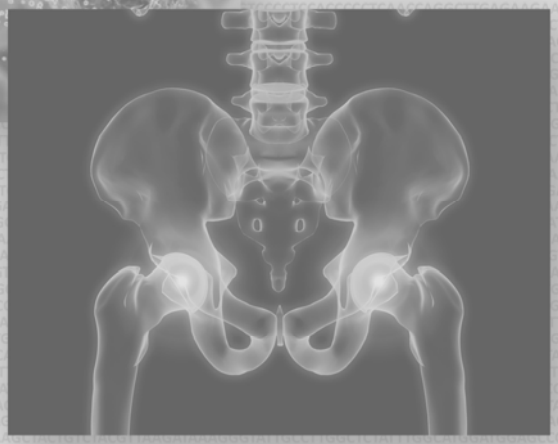

The role of plasma cytokine levels, CRP and selenoprotein S gene variation in OA.

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Abstract

Objective: Investigating the association between plasma levels of cytokines and chemokines, Selenoprotein S (*SELS*) gene variation and osteoarthritis (OA) subtypes.
Methods: The GARP study consists of 191 sibling pairs with symptomatic OA at multiple joint sites. We have measured plasma levels of 17 cytokines and chemokines and genetic variation at the *SELS* gene.
Results: Nine out of 17 serum markers could be assessed quantitatively, whereas eight markers were assessed qualitatively. Principal component analysis (PCA) on the quantitatively assessed markers and serum high sensitive C-reactive protein (S-HsCRP) revealed that three components underlie 61% of the total plasma variation. Three single nucleotide polymorphisms (SNPs) in the *SELS* gene revealed four common haplotypes, one of which, GAG (frequency 3.5%) showed significant association to an anti-inflammatory (P = 0.019) and acute phase related (P = 0.036) component. OA subtype analysis showed that one component (mainly representing chemokine variation) was significantly associated to hand OA and disc degeneration (P = 0.029 and P = 0.010 respectively) as well as a physical component score (PCS) (P = 0.042). The CRP related component also showed a strong association to the PCS (P = 0.007). *SELS* haplotypes showed no association to OA subtypes in the GARP study.
Conclusion: Genetic variation in the *SELS* gene associates to components representing inflammatory signaling. Another component, representing chemokine variation, showed association to hand OA and disc degeneration in the GARP study indicating chemokines may contribute to OA pathogenesis.

Introduction

Osteoarthritis (OA) is a common joint disease and an important cause of pain and disability in the general population. Elucidation of common pathways that are involved in the onset and progression of the disease will assist in the development of new drug targets and provide a better management of this disabling condition in the future. Several studies have shown that genetic factors play an important role in OA etiology[1,2]. Although OA is not regarded to be an inflammatory disease, there is increasing evidence for the involvement of an innate low grade systemic inflammatory component which may partly explain the genetic susceptibility[3-7]. More specifically, support for the hypothesis that local variation in cytokine levels in the joint may influence OA onset and progression is found in the catabolic effects of pro-inflammatory cytokines, and the protective effects of anti-inflammatory cytokines in articular cartilage[8,9]. Gene expression studies have shown that in OA cartilage several cytokines and chemokines are highly expressed[10-12]. The recent introduction of multiplexed cytokine assays facilitates sensitive measurements of different cytokines using small amounts of valuable sample material from different origins. This sensitive technique allows investigation of the basal levels of chemokines and cytokines in plasma in relation to the ongoing disease processes for diseases where, as in OA, no pronounced and obvious inflammatory component is present.
In a previous study, Curran *et al.* showed that subjects can be characterized by a high (proinflammatory) or low (noninflammatory) plasma cytokine profile depending on a common promoter single nucleotide polymorphism (SNP) -105G > A (rs28665122) in the

Selenoprotein S (*SELS*) gene. A significant association of this *SELS* SNP was observed specifically with higher plasma levels of tumor necrosis factor alpha (TNFα), interleukin beta (IL1b) and interleukin 6 (IL6)[13]. SELS is a widely expressed protein involved in maintaining the functional integrity of the endoplasmatic reticulum (ER) by participating in the removal of misfolded proteins and regulating the cellular redox balance. SELS inhibition by siRNA revealed that a functionally impaired ER leads to activation of numerous pro-inflammatory cytokines mediated by nuclear factor kb (NF-kb) activation[13]. In a cohort of patients with intestinal inflammation, there was no association to the *SELS* locus, however, the pro-inflammatory allele associated to a high serum CRP levels in Crohn Disease patients with active disease[14]. Finally, *SELS* polymorphisms associated with coronary heart disease and ischemic stroke[15]. These studies trigger the question whether *SELS* gene variation also influences inflammatory responses and the etiology of OA. Hence, we set off to map the plasma levels of 17 cytokines and chemokines by use of a multiplexed bead array system in subjects with symptomatic OA at multiple joint sites of the GARP study. We tested whether *SELS* gene variation influenced these inflammatory plasma mediators and OA, and whether these markers associated to OA subtypes and severity.

Materials and methods

The GARP study

The GARP study consists of 191 sibling pairs. All participants have symptomatic OA at multiple sites in the hand or OA at two or more joint sites of four joint sites examined[16]. Symptomatic OA was determined following the American College of Rheumatology recommendations[17-19] whereas radiographic OA (ROA) was scored according to Kellgren/Lawrence[20]. Details on the GARP OA phenotype and inclusion criteria can be found in previous publications[16]. Physical functioning was assessed with the physical component score (PCS), a subscore of the Dutch validated RAND 36-item Health Survey. This questionnaire covers health related aspects including social functioning, role limitations, mental health and vitality[21,22]. A higher score on the PCS indicates a better physical functioning. For the current study we used the proportionate ROA score based on the presence of ROA at each joint location and on the number of joints with ROA identical as described previously. In short, scores 0, 1 and 2 represent respectively no, uni- and bilateral hip and knee OA for these joint sites. The hand ROA score (0-2) represents subjects with, respectively, 0-2, 3-6, and ≥7 hand joints affected out of 20 scored. For spinal discus degeneration (DD) score (0-2) represents subjects with DD at respectively 0-2, 3-5 and ≥6 levels out of 11 levels scored[23]. Hand OA following the ACR criteria[17] was analyzed in addition to the ROA criteria. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols. Written informed consent as approved by the ethical committee was obtained from all subjects in the GARP study.

Serum and plasma collection and measurements

For each participant of the GARP study a morning serum and EDTA plasma sample was collected. Samples were processed within 4 h upon collection and stored at -80°C until measurement. Serum high sensitive C-reactive protein (S-HsCRP) was assayed earlier[24]. A 17-plex bead assay provided by Bio-RAD was used to measure plasma levels of cytokines

and chemokines on a Luminex platform. Intra assay variation was estimated at 6.2%. The standard protocol was adjusted using twice the indicated amounts of plasma sample in half the amount of sample buffer to increase signal in the measurements. On each plate 10-15% of samples were in duplicate, in which no inconsistencies were observed. For calculation of z-scores the average value of duplos was used as a single value. For cytokines and chemokines with less than 60% of the fluorescence levels above background noise, a qualitative measure of detectable vs not detectable level was used (qualitative analysis, QL). For cytokines and chemokines with more than 60% fluorescence levels above background noise, z-scores of fluorescence levels were calculated per plate (quantitative analysis, QT). In the principal component analysis (PCA) individuals with missing values in five or more out of nine cytokine/chemokines measurements were excluded (N = 13). For the analyzed individuals remaining missing values were given the specific marker mean score. Sample distribution was random per plate on a total of eight plates.

Genotyping

In the current study three SNPs of the *SELS* were genotypes selected from the original paper of Curran *et al.*[13]. SNP positions relative to translation start of the *SELS* gene are -105 (G > A, rs28665122), +3705 (G > A, rs4965814), and +6218 (A > G, rs9874). SNPs were measured using hME™ chemistry on a matrix assisted laser adsorption/ionization time-of-flight mass spectrometry (MALDI-TOF) Mass Spectrometer (Sequenom Inc., San Diego, CA, USA). Assays were designed using the Sequenom MassARRAY Assay Design software (version 3.1). Assay conditions were standard conditions as described earlier[25]. In addition to the genotypes obtained by the Sequenom for rs28665122, a Taqman assay (Applied Biosystems, Foster City, California, United States) was performed to deal with low genotyping success rates this SNP using the Sequenom technique. Primers used for the ABI-genotyping were: forward primer 5'GGGTCGGCCTGCGA and reverse primer 5'CTTCCGGTGCGCTCCTA, probes were 5'TGGCCGGGACCAC labeled with VIC and 5'TTGGCCA GGACCAC labeled with FAM. Assays were run on a 7900HT (Applied Biosystems) according to the manufacturers specifications. Genotypes of both techniques were used in addition to each other where necessary and were used as a control where both techniques provided reliable genotypes. No inconsistencies were observed between techniques for the reliable genotypes.

Statistical analysis

PCA was applied to reduce the correlated data of the cytokines and chemokines plasma levels. Subjects (N = 341) with available levels of S-HsCRP and with >4 out of nine quantifiable markers were entered in the PCA analysis. In PCA analyses random missing data (see Table 2) was replaced by mean values. Both empirical criteria (percentage of variance explained by factors and Eigen values > 1) and interpretability were used to determine the number of factors. We explored the interpretability of these factors after applying a Varimax rotation with Kaiser Normalization. The loading score of each variable onto the individual factors represents the contribution of that variable to the variance observed in the resulting factor. For analysis only individual variable contributions of >0.4 qualified for loading a component[26]. A factor loading represents the linear relationship (Pearson correlation under Varimax rotation) between a variable and a factor.

In order to assess the relationships between OA characteristics, genetic variation at the *SELS* gene and the clusters of cytokines and chemokines, a mixed model regression analyses was performed. To investigate the individual associations, subject specific regression scores of each extracted cluster were used as dependent variable and age, sex, BMI (Body Mass Index) and all OA subtypes as co-variables. OA features and subtypes tested were specific ROA scores as defined previously; knee (0-2), hip (0-2), hand (0-2) and spinal DD (0-2)[24] and the PCS derived from the SF36 questionnaire. Furthermore, for each linear mixed model analysis family identity numbers (representing family relations) as random variables in order to model the familial dependencies that might occur for the levels.

Genotype distributions of *SELS* SNPs were checked by use of the HWE program available at http://linkage.rockefeller.edu. Thesias V3.1 was used to assess linkage disequilibrium between the SNPs and to assess the expected haplotypic contribution to a mean quantitative measure in carriers of a specific haplotype[27]. Quantitative measures analyzed by use of Thesias are e.g., the subject specific regression scores as determined by the PCA analyses. In individuals the expected quantitative measure is determined by the contribution of the two carried haplotypes. The Thesias program allows for adjustments of co-variables but not for familial relationship. Chi square analyses were performed to test for association of individual SNPs or proportionate ROA scores to the qualitatively analyzed cytokines and chemokines. P-values are unadjusted to multiple testing. Analyses were done in SPSS version 14 (SPSS, Chicago, IL, USA) unless mentioned otherwise.

Results

Cytokine and chemokine measurements

Characteristics of the GARP study are displayed in Table 1. For all participants of the GARP study cytokine and chemokine levels were analyzed in a plasma sample by use of a 17-plex bead array system. Table 2 shows cytokines and chemokines measured and the percentage of samples with levels above detection limit. In nine instances (see Table 2) we were able to assess a semi quantitative measure by the use of z-scores reflecting relative plasma levels. For the remaining eight instances (see Table 2), plasma levels were analyzed in a qualitative matter (detectable vs not detectable).

Association analysis of cytokine/chemokine levels with OA features

Nine cytokines and chemokines and S-HsCRP were analyzed in a quantitative manner, the remaining eight cytokines and chemokines were qualitatively analyzed. In the quantitatively analyzed cytokine and chemokine z-scores we observed substantial correlations (Supplementary Table 1). In addition to the association between S-HsCRP and BMI shown previously24, Supplementary Table 1 also shows the frequently observed association between BMI and plasma IL6 levels. GARP subjects in the highest BMI quartile (BMI > 29.1 kg/m^2) had an OR of 1.7 (95% C.I. 1.01-2.82) to reside in the highest quartile of IL6 plasma levels (P = 0.042) when corrected for age, sex and familial relationship. To reduce the redundancy between the markers to more independent components in which these variables cluster, a PCA including all inflammatory markers for which a quantitative measure was available (see Table 2) and S-HsCRP was performed. Table 3 shows the three components that were extracted. The coefficients depicted in Table

3 explain how well each individual marker is represented within the clusters. The marker levels of IL2, IL6, Granulocyte CSF Q3 (GCSF) and IL10 loaded together on the first component, explaining 34.1% of the total variation in the GARP study.

Table 1. Study characteristics of the 382 patients with OA at multiple joint sites (GARP study).

	N (%)	***Mean (sd)***	***range***
Women	312 (81.7)	-	-
Age (years)	382 (100)	60.27 (7.54)	42.66 – 79.44
BMI (kgm^{-2})	379 (99.2)	27.00 (4.67)	19.10 – 46.48
S-HsCRP (mgl^{-1})	354 (92.7)	3.63 (5.43)	0.21 – 56.80
Clinical hand OA	271 (70.7)	-	-
PCS SF36	375 (98.2)	54.02 (21.10)	8.75-98.75
ROA Score[1]	**0**	**1**	**2**
Knee	232	90	60
Hip	275	56	51
DD	125	181	76
Hand	169	110	103

BMI body mass index, S-HsCRP serum high sensitive C-reactive protein, ROA radiographic osteoarthritis, DD discus degeneration, PCS physical component scale

[1]All subjects were affected by OA at multiple joint sites. The scores 0, 1 and 2 represent a proportionate OA score, as described earlier[24]. In genetic analysis hip and knee replacements (respectively 38 and 8) were considered as OA, numbers indicated are patients with diseased joints at sampling, or had replacements within the year prior to sampling)

Table 2. Cytokines and chemokines in the 17-plex bead assay with levels above background noise per cytokine and chemokine.

Cytokine / Chemokine	*N*	*Detectable (%)*	*Analysis*
Interleukin 1β	107	28.3	QL
Interleukin 4	26	6.9	QL
Interleukin 12	90	23.8	QL
Interleukin 13	172	45.5	QL
Interleukin 17	25	7.1	QL
Interferon γ	179	47.4	QL
Tumor Necrosis Factor α	165	43.4	QL
Granulocyte Monocyte CSF	144	37.8	QL
Interleukin 2	234	61.6	QT
Interleukin 5	222	58.4	QT
Interleukin 6	374	98.4	QT
Interleukin 7	274	72.1	QT
Interleukin 8	359	94.5	QT
Interleukin 10	327	86.1	QT
Granulocyte CSF	248	65.3	QT
MCP-1	377	98.9	QT
MIP-1β	375	98.4	QT

QL stands for a qualitative analysis, QT stands for a quantitative analysis, CSF stands for colony stimulating factor., MCP stands for Monocyte Chemotactic Protein, MIP stands for Macrophage Inflammatory Protein.

Table 3. Individual factor cytokine scores extracted by principal component analysis of 10 inflammatory markers measured in blood.

Component[1]	1	2	3
IL2	0.839		
IL6	0.815		
Granulocyte CSF	0.764		
IL10	0.594		
IL7	0.561		0.474
MCP-1		0.799	
IL8		0.799	
MIP-1β		0.765	
S-HsCRP			-0.747
IL5			0.585
Total variation explained	34.1%	15.7%	11.6%

[1]Extraction Method: Principal Component Analysis in which missing values were replaced by mean levels.
IL stands for Interleukin, CSF stands for colony stimulating factor, MCP stands for Monocyte Chemotactic Protein, MIP stands for Macrophage Inflammatory Protein, S-HsCRP stands for serum high sensitive C-reactive protein.

Component 2 is determined by three chemokines MCP (Monocyte Chemotactic Protein), IL8 and MIP (Macrophage Inflammatory Protein), explaining 15.7% of the variation, whereas the third component is determined by S-HsCRP, IL5 and IL7 explaining 11.6% of the variation. It should be noted that S-HsCRP has a negative value in the third component (Table 3), indicating that on average, within subjects there is an inverse relation between S-HsCRP levels and IL7 and IL5.

Subsequently the relationship between the three components as dependant variables and the presence of OA characteristics (Table 1) as co-variables was investigated by mixed model regression analysis. The upper section of Table 4 shows that component 2, consisting of chemokines IL8, MIP and MCP, has significant negative associations to hand ROA score (beta = -0.14 P = 0.039) and to disc degeneration ROA score (beta = -0.22 P = 0.005), independent of age, sex and BMI. This implies that subjects with high chemokine levels have lower hand ROA and DD scores. In addition, component 2 showed a similar association to subjects that had hand OA according to the ACR criteria (beta= -0.26; P = 0.024, data not shown). When analyzing the relationship of the components to the PCS derived from the SF36 a significant negative association of component 2 (P = 0.035) and a positive association to component 3 (P = 0.004) was observed, independent of sex, age and BMI (Table 4, lower section). This indicates that subjects with high chemokine levels experience more functional impairment whereas subjects with high IL7, IL5 and low S-HsCRP levels experience less functional impairment. The association of PCS to component 2, however, appeared not independent of the hand ROA scores (data not shown). In each of the mixed models significant associations with BMI were observed for component 1 (positive, P = 0.049), for component 2 (negative, $P < 1 \times 10^{-5}$) and component 3 (negative, $P < 1 \times 10^{-5}$). In the qualitatively analysis of the cytokines no significant associations were observed for OA subtypes (data not shown).

Table 4. Effect sizes (β) of the linear relationships between the extracted principal components (1-3) reflecting variation at plasma chemokines / cytokine levels and OA characteristics (ROA score, clinical symptoms) of the subjects of the GARP study sample.

OA characteristics	Components[1]		
Radiographic OA	**1**	**2**	**3**
Hip ROA score	0.079	0.098	0.035
Knee ROA score	-0.079	0.043	-0.088
Hand ROA score	0.030	-0.140*	-0.042
DD ROA score	0.002	-0.223*	-0.060
SF36 outcome	**1**	**2**	**3**
PCS	0.001	-0.006*	0.007**

[1]Component 1 contains IL2, GCSF, IL6, IL10 and IL7. Component 2 contains MCP, IL8, MIP and component 3 contains S-HsCRP (negative), IL5 and IL7. Data was analyzed using mixed model regression analyses with the components as dependent variable and as co-variables the joint specific ROA scores (top) or the PCS (bottom) in addition to age, sex and BMI. PCS stands for physical component score.
* $P < 0.05$ ** $P < 0.01$.

SELS gene variation, inflammatory parameters and OA

In the GARP study we could not confirm the previously reported association of rs28665122 with TNFα, IL1-b and IL6 plasma levels by Curran *et al.*[13].

The influence of the *SELS* SNPs on the cytokine and/or chemokine levels, as expressed by the three components, was investigated by haplotype analysis since high linkage disequilibrium was observed between the three *SELS* SNPs (D' > 0.8). As shown in Table 5, four common haplotypes with frequencies over one percent were observed similar to a Finnish population[15]. A significant association was observed between haplotype GAG (frequency 3.5%) and component 1 (P = 0.019). Since component 1 reflects variation in IL2, IL6, GCSF and IL10 levels this association indicates that carriers of this haplotype have higher levels of the cytokines in this component. Upon further investigation, this association appeared to be mainly driven by IL10 variation (univariate analysis P = 0.001). In addition, the GAG haplotype shows association to component 3 (P = 0.036) containing IL5, IL7 and inversely S-HsCRP. This association appeared to be mainly attributable to S-HsCRP levels in the component (univariate analysis P = 0.002). These associations were independent of BMI, age and sex. The fact that both components associate to the GAG haplotype indicates some interrelation between the haplotype and these components. We were not able to asses association between *SELS* SNPs or haplotypes and OA subtypes.

Table 5. Haplotype frequencies within the GARP study with their mean haplotypic contribution to the component scores as extracted from the data of 9 cytokines/chemokines and CRP.

Haplotype	Number (%)	C1[1,2] mean (95% C.I.)	C2[1,2] mean (95% C.I.)	C3[1,2] mean (95% C.I.)
GGA	638 (83.5)	-0.25 (-0.61 - 0.11)	-0.27 (-0.58 - -0.05)	0.85 (0.56 - 1.15)
AAG	81 (10.6)	-0.22 (-0.65 - 0.20)	-0.39 (-0.79 - 0.00)	0.83 (0.48 - 1.18)
GAG	27 (3.5)	0.68 (0.24 - 1.10)*	-0.23 (-1.06 - 0.61)	1.98 (1.35 - 2.61)*
GAA	10 (1.3)	-0.68 (-2.57 - 1.21)	-0.37 (-1.38 - 0.64)	0.92 (0.29 - 1.54)
other	8 (1.1)	-	-	-
Total	764 (100)			

* BMI adjusted *P*-value <0.05 for contribution of the haplotype GAG to the component score as compared to the other haplotypes as determined by THESIAS. C1 component 1, C2 component 2, C3 component 3. [1]Scores displayed are the expected haplotypic contribution (independent of the BMI effects) to the mean "principle component regression score" of subjects calculated by the Thesias program. In individuals the expected level is determined by the contribution of the 2 carried haplotypes. The THESIAS program does not allow correction for familial relationship. Alleles are in the following order of SNPs in the *SELS* gene -105 (rs28665122G>A), +3705 (rs4965814G>A), and +6218 (rs9874A>G). [2]Component 1 reflects variation of IL2, IL6, GCSF and IL10 levels, component 2 reflects variation of chemokines MCP, IL8 and MIP and component 3 reflects variation of HsCRP (negative), IL5 and IL7.

Discussion and conclusion

In inflammation driven diseases high circulating plasma levels of pro-inflammatory cytokines and S-HsCRP are present well above detection limits of current methods and readily used for diagnostic and prognostic purposes[28]. Cytokines, however, are known to exert their wide ranged actions also in very low concentrations. The recent introduction of multiplexed cytokine assays facilitates simultaneous measurements of multiple cytokines. However, the described absence of a large scale upregulation or strong association of any of the measured cytokines or chemokines in the plasma of subjects with familial OA at multiple joint sites indicates that, in blood plasma, these markers are not sufficiently suitable to monitor the ongoing OA process. Synovial fluid measurements might better reflect the ongoing disease process since it better reflects the cytokine activities near the site where the disease is mainly active[29]. PCA analyses of cytokine/chemokines measurements revealed 3 components. component 1 reflects variation at IL2, IL6, GCSF and IL10 levels, component 2 reflects variation at chemokines MCP, IL8 and MIP and component 3 SHsCRP (negative), IL5 and IL7. The components seem to reflect different ongoing (patho) physiological processes identified by subjects underlying the components. Component 1 may be classified as a marker of ongoing anti-inflammatory signaling based on the strong involvement of IL10, whereas component 2 shows chemokine signaling and component 3 reflects more acute phase related signaling. The observed (Supplementary Table 1) and known correlation of IL6 to S-HsCRP is, with the current setting in the PCA (Eigen values > 1), not reflected in the components since together they do not explain sufficient amount of the variation.

We found a significant negative association for component 2 to hand OA and DD as well as to the SF36 derived PCS. This indicated that especially the subjects that exhibit high

functional impairment and have low hand ROA scores have high chemokine levels. It should be noted that, by definition of the GARP selection criteria, subjects of the GARP study with low hand or disc ROA scores have OA at other joint sites. We could not, however, attribute the negative association of hand and disc ROA to positive effects caused by these other joint sites. Given these results it appears the higher levels of chemokines act protective in hand and disc OA among subjects of the GARP study. The strong association of the PCS to component 3 including S-HsCRP might reflect impairment of physical functioning mediated or reflected by the individual markers in this component, the observed effects are independent of BMI and ROA status. Due to the relative large amount of missing values in the IL5, IL7 and GCSF data we may have missed specific associations with these markers.

Previously, it has been shown that there is a major upregulation of chemokines in human OA affected cartilage[12]. Our analysis show that, amongst subjects of the GARP study, this upregulation might be less pronounced in subjects with hand OA and disc degeneration of the spine as compared to the other subjects, when correcting for all involved joint sites. This may reflect a different pathophysiological process underlying hand OA and disc degeneration as compared to knee and hip OA. This needs to be further explored in other cohorts of OA patients and especially using control samples.

Given the earlier found associations of *SELS* SNPs to inflammatory factors measured in blood[13], we expected to find associations of cytokines and chemokines especially to rs28665122, however, no direct associations of these levels or presence of these cytokines and chemokines were observed for variation at the *SELS* gene. A recent paper of Seiderer *et al.*[14] also showed no confirmation of the association for rs28665122 to cytokine levels in a study including patients with intestinal inflammation. As compared to the study in intestinal inflammation patients, however, Curran *et al.* used a more sensitive method of measuring cytokines. Seiderer *et al.* did observe an association of the pro-inflammatory allele to higher serum CRP levels in a subgroup of Crohn disease patients with higher signs of disease activity[14]. In our study, possibly the association to either cytokines or S-HsCRP for rs28665122 is not observed due to a smaller sample size or upregulation of these cytokines by the ongoing disease processes is not sufficient to show the genotype effect as observed by Seiderer *et al.*. However, haplotype association analyses revealed a specific *SELS* haplotype (GAG, 3.5%) significantly associated to components of increased IL10 blood levels and decreased S-HsCRP levels, confirming that the *SELS* gene variation may interfere with or affect the homeostasis of the inflammatory pathways.

In the PCA individuals with missing values in five or more out of nine cytokine/chemokines measurements were excluded (N = 13). For the analyzed individuals remaining missing values were given the specific marker mean score. Although we could not readily detect the cause of values being missing most likely these occurred due to bad sample quality or assay errors and less likely due to individuals being out of range. Performing PCA using a list wise case selection (using only cases with all markers available), replacing missing values by the lowest observed value, imputation of the missing values by use of regression analysis or by use of multiple imputation using a winMICE implemented EM algorithm[30] did not affect formation of components or subsequent associations (data not shown). The components reported should be considered robust. The effect of the familial dependencies on the component formation is considered to be minimal as the total number of pairs in the dataset cancels out possible intra sibling pair

correlations. This is strengthened by the results of an analysis of the data using only one member per sibling pair (data not shown) which shows highly similar component and scores.

Moreover, by excluding individuals with over four out of nine missing values we may have excluded individuals with a particular low-inflammatory profile. Finally, missing values in the qualitatively analyzed cytokines may likewise have been subject to possible misclassification of individuals due to bad sample quality, assay errors or measurement problems.

Since OA patients are likely to use drugs which alter the immune system we explored whether the use of NSAIDs significantly influenced our results. In a split analysis for use vs no use of NSAIDs the formation of the components in the PCA showed no major changes, except for the third component in NSAID users where IL5 and IL7 disappeared. The described subsequent associations remained present in both separate datasets (data not shown).

In earlier investigations of the GARP study, we have found that S-HsCRP levels are independently associated to a *CRP* haplotype[7,13] and by the occurrence of the closely related factors knee ROA, BMI and high WOMAC scores[24]. In the current study we show that a *SELS* haplotype is additionally influencing the S-HsCRP level. When we fitted a mixed model with S-HsCRP as dependent variable and as co-variables the *CRP* haplotype H7/8, *SELS* haplotype GAG, and the factor representing knee ROA, BMI and WOMAC24, it was shown that all three consistently and independently influence the S-HsCRP level. In this model, the *SELS* haplotype (GAG) significantly decreased (beta = -0.42; $P = 1.9 \times 10^{-5}$) S-HsCRP levels in carriers whereas the S-HsCRP haplotype H7/8 (beta = 0.14; P = 0.08) and the PCA component (beta = 0.14; $P = 1.6 \times 10^{-9}$) increased S-HsCRP level.

The strength of the GARP study lies in the availability of extended clinical and radiological data of OA features for four joint sites. Furthermore, demographic data for the participants is available, as well as additional familial information, a range of biological fluids and DNA. A downside for this study is, however, that the sample size is relatively small for genetic studies and no synovial fluid samples and control samples of healthy individuals are available for the cytokine measurements in this study.

The analysis of a range of cytokines as measured in blood has not shown any strong associations of one single cytokine to OA features; however, a component comprised of several chemokines did show association to OA in smaller joints and PCS. Another component which has strong involvement of S-HsCRP shows a highly significant association to this PCS, indicating physical impairment might be reflected or mediated by the markers in this component, independent of presence of specific ROA subtypes. Future measurements of chemokines and related signaling proteins in synovial fluid may shed more light on the origin of this association. Long term effects of lower circulating SHsCRP, cytokine or chemokine levels might be reflected by OA progression. Upcoming follow up data in the GARP study will reveal whether these inflammatory parameters associate to further active progression of OA.

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References

1. Ikegawa S. New gene associations in osteoarthritis: what do they provide, and where are we going? *Curr Opin Rheumatol* 2007; 19(5):429-434.
2. Valdes AM, Loughlin J, Oene MV, Chapman K, Surdulescu GL, Doherty M *et al.* Sex and ethnic differences in the association of ASPN, CALM1, COL2A1, COMP, and FRZB with genetic susceptibility to osteoarthritis of the knee. *Arthritis Rheum* 2007; 56(1):137-146.
3. Botha-Scheepers SA, Watt I, Slagboom E, de Craen AJ, Meulenbelt I, Rosendaal FR *et al.* Innate production of Tumor Necrosis Factor-{alpha} and Interleukin-10 is associated with radiological progression of knee osteoarthritis. *Ann Rheum Dis* 2007.
4. Pelletier JP, Martel-Pelletier J, Abramson SB. Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. *Arthritis Rheum* 2001; 44(6):1237-1247.
5. Riyazi N, Slagboom E, de Craen AJ, Meulenbelt I, Houwing-Duistermaat JJ, Kroon HM *et al.* Association of the risk of osteoarthritis with high innate production of interleukin-1beta and low innate production of interleukin-10 *ex vivo*, upon lipopolysaccharide stimulation. *Arthritis Rheum* 2005; 52(5):1443-1450.
6. Spector TD, MacGregor AJ. Risk factors for osteoarthritis: genetics(1). *Osteoarthritis Cartilage* 2004; 12 Suppl A:39-44.
7. Bos SD, Suchiman HE, Kloppenburg M, Houwing-Duistermaat JJ, Hellio Le Graverand MP, Seymour AB *et al.* Allelic variation at the C-reactive protein gene associates to both hand osteoarthritis severity and serum high sensitive CRP levels in the GARP study. *Ann Rheum Dis* 2007; 67(6):877-879.
8. Goldring MB. Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. *Best Pract Res Clin Rheumatol* 2006; 20(5):1003-1025.
9. Goldring MB, Goldring SR. Osteoarthritis. *J Cell Physiol* 2007; 213(3):626-634.
10. Appleton CT, Pitelka V, Henry J, Beier F. Global analyses of gene expression in early experimental osteoarthritis. *Arthritis Rheum* 2007; 56(6):1854-1868.
11. Borzi RM, Mazzetti I, Marcu KB, Facchini A. Chemokines in cartilage degradation. *Clin Orthop Relat Res* 2004;(427 Suppl):S53-S61.
12. Attur MG, Dave M, Akamatsu M, Katoh M, Amin AR. Osteoarthritis or osteoarthrosis: the definition of inflammation becomes a semantic issue in the genomic era of molecular medicine. *Osteoarthritis Cartilage* 2002; 10(1):1-4.

13. Curran JE, Jowett JB, Elliott KS, Gao Y, Gluschenko K, Wang J *et al.* Genetic variation in selenoprotein S influences inflammatory response. *Nat Genet* 2005; 37(11):1234-1241.
14. Seiderer J, Dambacher J, Kuhnlein B, Pfennig S, Konrad A, Torok HP *et al.* The role of the selenoprotein S (*SELS*) gene -105G>A promoter polymorphism in inflammatory bowel disease and regulation of *SELS* gene expression in intestinal inflammation. *Tissue Antigens* 2007; 70(3):238-246.
15. Alanne M, Kristiansson K, Auro K, Silander K, Kuulasmaa K, Peltonen L *et al.* Variation in the selenoprotein S gene locus is associated with coronary heart disease and ischemic stroke in two independent Finnish cohorts. *Hum Genet* 2007; 122(3-4):355-365.
16. Riyazi N, Meulenbelt I, Kroon HM, Ronday KH, Hellio Le Graverand MP, Rosendaal FR *et al.* Evidence for familial aggregation of hand, hip, and spine but not knee osteoarthritis in siblings with multiple joint involvement: the GARP study. *Ann Rheum Dis* 2005; 64(3):438-443.
17. Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein D, Brandt K *et al.* The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hand. *Arthritis Rheum* 1990; 33(11):1601-1610.
18. Altman R, Alarcon G, Appelrouth D, Bloch D, Borenstein D, Brandt K *et al.* The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. *Arthritis Rheum* 1991; 34(5):505-514.
19. Altman RD, Asch E, Bloch D. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. *Arthritis Rheum* 1986; 29:1039-1049.
20. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. *Ann Rheum Dis* 1957; 16(4):494-502.
21. Hays RD, Sherbourne CD, Mazel RM. The RAND 36-Item Health Survey 1.0. *Health Econ* 1993; 2(3):217-227.
22. Ware JE, Kosinski M. Interpreting SF-36 summary health measures: a response. *Qual Life Res* 2001; 10(5):405-413.
23. Meulenbelt I, Kloppenburg M, Kroon HM, Houwing-Duistermaat JJ, Garnero P, Hellio Le Graverand MP *et al.* Urinary CTX-II levels are associated with radiographic subtypes of osteoarthritis in hip, knee, hand, and facet joints in subject with familial osteoarthritis at multiple sites: the GARP study. *Ann Rheum Dis* 2006; 65(3):360-365.
24. Meulenbelt I, Kloppenburg M, Kroon HM, Houwing-Duistermaat JJ, Garnero P, Hellio-Le Graverand MP *et al.* Clusters of biochemical markers are associated with radiographic subtypes of osteoarthritis (OA) in subject with familial OA at multiple sites. The GARP study. *Osteoarthritis Cartilage* 2007; 15(4):379-385.
25. Min JL, Meulenbelt I, Riyazi N, Kloppenburg M, Houwing-Duistermaat JJ, Seymour AB *et al.* Association of matrilin-3 polymorphisms with spinal disc degeneration and osteoarthritis of the first carpometacarpal joint of the hand. *Ann Rheum Dis* 2006; 65(8):1060-1066.
26. Stevens J. Applied multivariate statistics for the social sciences. *Hillsdale (NJ): Lawrence Erlbaum Associates* 1986.

27. Tregouet DA, Escolano S, Tiret L, Mallet A, Golmard JL. A new algorithm for haplotype-based association analysis: the Stochastic-EM algorithm. *Ann Hum Genet* 2004; 68(Pt 2):165-177.
28. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 2007; 7(6):429-442.
29. Hulejova H, Baresova V, Klezl Z, Polanska M, Adam M, Senolt L. Increased level of cytokines and matrix metalloproteinases in osteoarthritic subchondral bone. *Cytokine* 2007; 38(3):151-156.
30. Van Buuren S, Oudshoorn CGM. Multivariate Imputation by Chained Equations: MICE V1.0 User's manual. TNO Report PG/VGZ/00.038. Leiden: TNO Preventie en Gezondheid. <http://web.inter.nl.net/users/ S.van.Buuren/publications/MICE V1.0 Manual TNO00038 2000.pdf>; 2007.

Supplementary Table 1. Spearman's rank correlations with P-values between chemokine, cytokines, HsCRP and BMI among subjects of the GARP study.

	P-values										
	HsCRP	IL2	IL5	IL6	IL7	IL8	IL10	GCSF	MCP	MIP	BMI
HsCRP		7.1×10^{-1}	2.3×10^{-1}	1.7×10^{-4}	3.8×10^{-1}	1.4×10^{-2}	6.9×10^{-1}	1.5×10^{-2}	4.2×10^{-1}	6.0×10^{-1}	1.0×10^{-6}
IL2	-0.03		9.1×10^{-1}	1.0×10^{-6}	1.0×10^{-6}	2.1×10^{-2}	1.0×10^{-6}	1.0×10^{-6}	0.09	0.01	4.3×10^{-1}
IL5	-0.08	0.01		1.3×10^{-2}	4.0×10^{-4}	5.8×10^{-2}	3.2×10^{-5}	1.5×10^{-1}	1.3×10^{-2}	3.1×10^{-3}	4.5×10^{-1}
IL6	0.20	0.58	0.17		1.0×10^{-6}	1.0×10^{-6}	1.0×10^{-6}	1.0×10^{-6}	1.0×10^{-6}	1.0×10^{-6}	2.0×10^{-3}
IL7	-0.05	0.38	0.27	0.32		7.9×10^{-3}	1.0×10^{-6}	3.8×10^{-4}	1.2×10^{-4}	1.4×10^{-6}	4.4×10^{-1}
IL8	0.13	0.16	0.13	0.33	0.17		1.0×10^{-6}	2.7×10^{-2}	1.0×10^{-6}	1.0×10^{-6}	6.6×10^{-1}
I10	0.02	0.36	0.29	0.47	0.33	0.42		1.3×10^{-5}	1.0×10^{-6}	1.0×10^{-6}	6.8×10^{-1}
GCSF	0.16	0.42	0.11	0.38	0.26	0.14	0.28		5.0×10^{-2}	9.0×10^{-2}	3.7×10^{-1}
MCP	0.04	0.11	0.17	0.27	0.23	0.49	0.37	0.12		1.0×10^{-6}	2.4×10^{-1}
MIP	-0.03	0.17	0.20	0.28	0.29	0.49	0.38	0.11	0.50		1.5×10^{-1}
BMI	0.36	0.05	-0.05	0.16	-0.05	0.02	-0.02	0.06	0.06	0.08	

Spearman's correlations