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Clusters within a wide spectrum of biochemical markers for osteoarthritis: data from CHECK, a large cohort of individuals with very early symptomatic osteoarthritis

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SUMMARY

Objective: To assess a wide spectrum of biochemical markers (biomarkers) in a large cohort of individuals with (very) early symptomatic knee and/or hip osteoarthritis (OA). Secondly, to investigate associations between biomarkers and between biomarkers and demographics to demonstrate validity of the obtained dataset and further investigate the involvement and/or role of these biomarkers in OA.
Design: Fourteen biomarkers (uCTX-II, uCTX-I, uNTX-I, sCOMP, sPIIANP, sCS846, sC1,2C, sOC, sPINP, sHA, sPIIINP, pLeptin, pAdiponectin, pResistin) were assessed by ELISA or RIA in CHECK (Cohort Hip and Cohort Knee), a 10-year prospective cohort of 1,002 individuals with early symptomatic knee and/or hip OA.
Results: Quality controls revealed that gathered data were technically reliable. The majority of biomarkers showed relevant associations with demographic variables, which were expectedly different between genders and/or menopausal status for some. Principal component analysis enabled identification of five clusters, consecutively designated as 'bone-CTX-II', 'inflammation', 'synovium', 'C1,2C-adipokines', and 'cartilage synthesis' cluster. Notably, uCTX-II clustered with biomarkers of bone metabolism, while sCOMP clustered with biomarkers of synovial activity.
Conclusions: The identified clusters extended knowledge on individual biomarkers from mostly smaller studies as did the observed associations between biomarker levels and demographics, from which validity of our data was deduced. uCTX-II may not only reflect articular cartilage but also bone metabolism and sCOMP may reflect synovial rather than cartilage metabolism. Major involvement of adipokines in joint metabolism was not identified.

Introduction

Osteoarthritis (OA) is a slowly-progressive, degenerative, low-grade inflammatory joint disease that is commonly diagnosed in a late stage when significant joint damage has already occurred. Tools for early-stage diagnosis may create an opportunity for disease modifying treatment modalities. Development of such treatments is hampered by a poor understanding of OA pathogenesis, the slowly-progressive character of OA, and insensitive monitoring methods that necessitate long-term and large-scale clinical trials[1]. Biochemical markers (biomarkers) could be valuable tools for early-stage OA diagnosis, follow-up of therapeutic response, prediction of disease course, discrimination between OA subtypes, and/or gaining further insight into OA pathogenesis. As such, they might ultimately lead to improved diagnostic and therapeutic modalities for OA.

Current knowledge of biomarkers is mainly based on data from small cohorts and/or only one or a few simultaneously assessed biomarkers[2]. Investigating small cohorts decreases statistical power and increases the chance of coincidental findings, probably partly explaining the inconsistent performance of biomarkers in literature[1,2]. Moreover, assessing only one or a few biomarkers is

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discordant with the current view on OA as a complex joint disease. Accordingly, the potential value of simultaneous assessment of multiple biomarkers is increasingly recognized[3].

Here we report on a large-scale assessment of 14 putative biomarkers for OA in CHECK (Cohort Hip and Cohort Knee), a 10-year prospective cohort of 1,002 participants with (very) early symptomatic OA of knee and/or hip. Having a valid biomarker dataset in addition to the many longitudinal radiographic and clinical data that are available for all CHECK participants would allow thorough investigation of cross-sectional and predictive associations between biomarkers and OA parameters in (very) early-stage knee and hip OA. In the current report, we discuss the approach in this comprehensive biomarker assessment. We then demonstrate its validity by investigating associations between biomarkers and demographic variables and exploring mutual associations between biomarkers. In addition, we extend hypotheses on the biological backgrounds of some of the biomarkers.

Method

Cohort and sample collection

Biomarkers were assessed in baseline urine, serum, and plasma samples of CHECK[4]. CHECK is a 10-year prospective cohort study of 1,002 individuals aged 45–65 years with pain and/or stiffness of one and/or both knee(s) and/or hip(s). They had never or not longer than 6 months ago consulted a physician for these symptoms. Participants with any other pathological condition (e.g., other rheumatic disease or joint trauma) that could explain the symptoms were excluded. All participants are broadly characterized throughout follow-up: clinically through regular clinical examinations and questionnaires, radiographically through knee and hip radiographs at predefined time points (at baseline and after 2, 5, 8, and 10 years), and biochemically through collection of plasma, serum, and urine samples at the time of radiography. Ultimately, integration of all these aspects will aid to identification of factors that play a key role in the development and progression of OA. CHECK is conducted according to the declaration of Helsinki (as revised in 2000) and received approval from the ethics committee of each of the involved medical centers. Written informed consent was obtained from all participants before inclusion.

At baseline, participants (79.0% female) were aged 56 ± 5 years [mean ± SD (standard deviation)] and had a BMI of 25.5 kg/m^2 (median, 25–75% percentile 23.3–28.4). Women were designated as postmenopausal when menstruation had stopped >12 months before their baseline visit. When menopausal status could not be defined because of previous hysterectomy or bilateral ovariectomy, oral anticonceptive use, or missing data, women aged >55 years were designated as postmenopausal and menopausal status of women aged 45–55 years was designated as undetermined. Women using any sex hormonal therapy were also not included in analyses including menopausal status. As such, 142 (17.9% of all women) women were designated as premenopausal, 475 (60.0%) postmenopausal, and 175 (22.1%) undetermined.

Of those participants with complete baseline radiographic data for knees and hips (n = 989), 24.6% showed only knee OA, 13.4% showed only hip OA, 7.2% showed both knee and hip OA, while 54.8% showed OA of neither knee nor hip (OA defined as K&L grade ≥1). Seventy-six percent of the participants with knee complaints and 24% of the participants with hip complaints fulfilled the clinical ACR criteria for the classification of OA.

Sample tubes for plasma, serum, and urine were labeled with participant identification numbers at the time of collection. Non-fasted serum, plasma, and second morning void urine samples were collected between 8 and 12 a.m. and transported within 4 h by courier to the central laboratory (urine and plasma on ice, serum at ambient temperature) where they were processed according to protocols, regularly audited by a central coordinator. Gel serum separation tubes were left at room temperature for at least 1 h to allow clotting and centrifuged (15 min, 1,000–1,200 g). EDTA plasma tubes were centrifuged (15 min, 2,000 g, 4°C) and plasma separated. Urine was collected in standard containers. All samples were aliquoted and stored in cryotubes at −80°C.

Biomarkers

Eleven biomarkers of joint metabolism (Table I) were selected on the basis of their performance as described in literature[2] and so that they would represent complete joint metabolism, representing anabolic as well as catabolic pathways in cartilage, (subchondral) bone, and synovial tissue[5] according to current understanding. Since there is ambiguous data on the involvement of adipokines in the pathogenesis of OA[6–9], we additionally assessed three adipokines (Table I).

Assessment procedure

Biomarkers were assessed by commercially available ELISA or RIA assays (Table I), according to manufacturer instructions. As such, kits were not extensively (re)validated. To circumvent inter-batch variability, all assay kits were purchased from single

Table I
Simplified overview of the spectrum of biomarkers and adipokines that were assessed in CHECK. The targeted processes that are indicated per biomarker are primarily based on a review article by Garnero *et al.*[5]. However, it should be emphasized that most biomarkers cannot be considered joint specific and/or molecular validity is not definitely demonstrated for most of them. Reference numbers refer to references in the main text

Targeted OA process	Biochemical marker	Assay kit*
Cartilage degradation	uCTX-II[24,37–39]	Urine Cartilaps EIA, Immunodiagnostic systems Ltd., Boldon, UK
	sCOMP[31,46]	Anamar Med AB, Göteborg, Sweden
Cartilage synthesis	sPIIANP[38,39]	Millipore Corp, Billerica, MA, US
	sCS846[47]	IBEX, Montreal, Canada
(Subchondral) bone degradation	uCTX-I[19,20,22,23,25]	Urine Crosslaps EIA, Immunodiagnostic systems Ltd., Boldon, UK
	uNTX-I[20,21]	OSTEOMARK NTx Urine, Wampole Laboratories, Princeton, NJ, US
(Subchondral) bone synthesis	sPINP[19–21,25]	UniQ, Orion Diagnostica, Espoo, Finland
	sOC[19,20,22,25,26]	N-MID Osteocalcin ELISA, Immunodiagnostic systems Ltd., Boldon, UK
Synovial tissue synthesis	sPIIINP[41]	UniQ, Orion Diagnostica, Espoo, Finland
Synovial tissue degradation and/or activity	sHA[42]	Corgenix Inc, Westminster, CO, US
Cartilage and (subchondral) bone degradation	sC1,2C[7]	IBEX, Montreal, Canada
Adipokines	pLeptin[8]	BioVendor, Modrice, Czech Republic
	pAdiponectin[7]	BioVendor, Modrice, Czech Republic
	pResistin[9]	BioVendor, Modrice, Czech Republic

* All ELISAs, except for UniQ PINP and PIIINP RIAs.

batches (14 plates per batch). Commercial independence was valued highly when purchasing the assay kits.

Triplicate assessment in a subset of at least 50 samples showed adequate precision of singlicate as compared to triplicate assessment (i.e., biomarker levels in the first samples of triplets as compared with mean biomarker levels in these triplets); intraclass coefficients[10] were high (≥0.832) [Table SI]. Assessment was performed in singlicate for all biomarkers in all samples.

Unexpectedly, the influence of multiple freeze–thaw cycles and storage time appeared to be not or only minimally investigated for the selected biomarkers[11–18]. Therefore, it was decided that biomarkers needed to be assessed on the day of thawing and that samples needed to be thawed only once. This necessitated simultaneous assessment of multiple biomarkers per sample by several technicians. Each technician worked on (a) specific biomarker(s) so that each biomarker was assessed in all samples by the same technician. Time plans were set up so that technicians were able to perform several assay kits simultaneously. All technicians were skilled in ELISA or RIA. One supervisor (NJ) coordinated logistics and monitored continuity of procedures throughout the assessment period. Each transition of samples or data took place according to predefined templates and was always checked by at least two persons.

All samples were assessed within maximum 4 h after thawing. Stability of the biomarkers during these 4 h was assumed on the basis of manufacturer data and on the expected inherent stability of (the epitopes of) matrix proteins. Prior to biomarker assessment, samples were centrifuged (10 min, 2,000 *g*). All samples were equally diluted for each biomarker. Of the approximately 14,000 assessments that were performed in total, a small minority showed biomarker concentrations that were outside standard curves (Table II). When these samples were re-assessed in the original as well as adapted dilutions clear indications for unpredictable, non-linear dilution effects were observed (data not shown). Therefore, concentrations that were assessed in adapted dilution were considered invalid. Thorough investigation of this issue (e.g., using other than the supplied diluents) was not performed, because of the very small number of samples concerned per biomarker. Since omitting these samples from further analyses could introduce a bias, samples that were too high or low were arbitrarily set on 120% of the maximum or 80% of the minimum biomarker level that was assessed in participant samples, respectively. Sensitivity analyses without these arbitrarily set levels and with too high and low levels set on 100% of maximum and minimum values were, and will always be, performed.

Blank samples (assay buffer) that were incorporated at predefined positions in between samples were all at their predefined positions at the time of microplate reader assessment, indicating that no major pipetting shifts had taken place. Internal control samples as supplied by manufacturers (not available for sC1,2C and sCS846) were incorporated in duplicate in each assay plate. Of them, 87.1% fell within manufacturers' ranges (data not shown). Aberrant internal control samples were only minimally outside reference ranges and in these cases the other control samples in that plate were within the specified ranges. The higher internal control samples of uNTX-I were consistently well below the provided reference range. Although this possibly indicated an aberrant course of the standard curve in the higher range, only a minority of the participants' samples showed uNTX-I concentrations in this range and data were considered acceptable.

Urine, serum as well as plasma standard samples were self-created by pooling samples from random OA outpatients and frozen in aliquots. One standard sample aliquot was freshly thawed on each assessment day and included in triplicate at predefined positions in all assay plates of that day. These standard sample data were used to quantify precision of the assessments.

Urinary creatinine concentrations were assessed in an automated kinetic assay (UniCel® DxC 800 Synchron® Clinical System, Beckman Coulter). hsCRP was assessed in all serum samples in an automated nephelometric assay (BN™ II analyzer, Siemens).

For sC1,2C and sCS846 it was noticed that concentrations within assay plates showed gradients from the first to the last pipetted sample (Spearman correlation analysis: r_s between −0.516 and −0.263, $P < 0.023$ in eight of 14 plates). Despite thorough analyses in consultancy with the involved manufacturer, no definite cause for this phenomenon could be identified.

Statistics

Assessment precision was expressed as the coefficient of variation (CV%, SD/mean value * 100%). Intra-plate CV%'s were calculated from the triplicate standard sample assessments that were performed per assay plate and averaged for all plates. Inter-plate CV %'s were calculated from the mean standard sample biomarker

Table II
Overview of numbers of successfully assessed samples, missing samples, and assessments outside standard curves (too low and/or too high) as well as biomarker levels in the successfully assessed samples

	N	Missing	Too low	Too high	Concentration (median (25–75% perc))	Intra-plate CV%	Inter-plate CV%	Between-day CV%
uCTX-II	956	38	8		193 (132–281) ng/mmol	10.0	9.3	12.4
sCOMP	960	42			8.5 (7.2–9.9) U/l	5.0	4.0	4.2
sPIIANP	960	39		3	1,385 (1,087–1,771) ng/ml	15.8	7.0	15.7
sCS846	963	39			70 (54–88) ng/ml	21.5	16.9	15.3
uCTX-I	954	38		10	152 (100–225) μg/mmol	9.7	6.1	2.7
uNTX-I	964	38			37 (28–51) nM BCE/mmol	14.9	6.6	10.7
sPINP	962	39		1	42 (32–56) ng/ml	4.4	4.5	6.2
sOC	962	38	1	1	13 (10–17) ng/ml	3.4	4.1	4.3
sPIIINP	957	39	6		4.1 (3.5–4.9) ng/ml	5.4	3.2	7.2
sHA	952	38	12		27 (17–43) ng/ml	15.1	13.0	17.3
sC1,2C	958	40		4	0.17 (0.14–0.22) μg/ml	19.3	13.0	31.3
pLeptin	940	21		41	11.3 (6.2–19.4) ng/ml	7.8	5.7	7.0
pAdiponectin	972	21		9	9.8 (7.3–14.0) μg/ml	18.9	14.3	9.0
pResistin	981	21			3.5 (3.0–4.3) ng/ml	7.1	3.9	2.5

Furthermore, intra-plate, inter-plate and between-day variability as calculated from repeated standard sample assessment in all assay plates are demonstrated.
uCTX-II, uCTX-I, uNTX-I, sCOMP: three standard samples per plate, four plates per day, four days.
sPIIANP, sCS846, sC1,2C, sOC, sHA: three standard samples per plate, two plates per day, nine days.
sPINP, sPIIINP: two standard samples per plate, two plates per day, eight days.
pAdiponectin, pLeptin, pResistin: three standard samples per plate, three plates per day, five days.
CV% (SD/mean * 100%).

levels that were assessed in each of the plates per day and averaged for all days. Between-day CV% was calculated from the mean biomarker levels in the standard samples that were assessed at each of the days.

Principal component analysis was performed to enable identification of clusters of interrelated biomarkers (components) within the spectrum of biomarkers. Biomarker levels were logarithmically transformed to obtain normal distributions. Direct Oblimin oblique rotation of the factor loadings, allowing for correlation among factors, was performed since the biomarkers were expected to originate from distinct but interrelated processes in the OA joint(s).

Associations between biomarker levels and demographic variables were investigated by multiple linear regression analysis. In each model one of the biomarkers was used as dependent variable, with demographics as independent variables. Biomarker levels and BMI were logarithmically transformed to obtain normally distributed variables and, thereby, residuals.

Modification of the association of age and BMI with biomarker levels by gender and/or menopause (e.g., different association of age with biomarker levels between genders) was investigated through interaction terms. When interaction terms appeared to be statistically significant, now defined as $P < 0.200$, analyses were repeated separately in gender or menopause subgroups for the biomarker concerned (i.e., stratified analyses).

To facilitate direct comparison between regression coefficients of variables and models, coefficients are expressed as standardized betas. Standardized betas represent the number of SDs that the outcome will change as a result of one SD change in the predictor and are therefore independent of the units of measurement of the variables and can vary between −1 and 1.

All statistical analyses were performed using SPSS 15.0. Statistical significance was defined as $P < 0.05$, unless stated otherwise.

Results

The numbers of participants for whom biomarker data were and were not obtained are tabulated in Table II. Missing data were due to absent sample tubes (lost at the stage of sample collection, storage, and/or recollection) or too small sample volumes to perform all assessments. Data represented here are inclusive of the arbitrary biomarker levels for samples outside standard curves (80% minimum, 120% maximum). Results were similar between analyses with and without these arbitrary biomarker levels and when outliers were alternatively set at 100% of minimum and maximum biomarker levels (i.e., sensitivity analysis) (data not shown).

Intra-plate, inter-plate, and between-day CV%'s for all biomarkers are tabulated in Table II. Especially the assessment of sC1,2C and sCS846 showed low precision (i.e., high CV%), probably related to the aforementioned technical issues. sHA, sPIIANP and pAdiponectin also showed considerable variability, but otherwise unremarkable quality controls (e.g., internal control samples, blank samples, gradients, data not shown).

Principal component analysis was performed to investigate mutual associations between biomarkers, adipokines, hsCRP, and ESR. When all participants were included in the analysis, all variables that were introduced in the model showed communalities ≥0.371, indicating that they all shared considerable variance (i.e., ≥37.1%) with any other variable(s) and including them in the analysis was appropriate. Five components had 'eigenvalues' ≥1, with the fifth component having an 'eigenvalue' of only 1.143 (Table IIIA and online supplementary data). Accordingly, the point of inflexion of the scree plot indicated that four components would represent the variation in the dataset most optimally (i.e., most optimal balance between complexity of the model and reflection of the variation in the dataset). Therefore, also a (forced) four-component model was investigated (Table SII). This time, sCS846 and sPIIANP that were already in the fifth component of the first model expectedly had very low communalities of 0.070 and 0.148, respectively (i.e., their variation could not be explained adequately by this model). After exclusion of sCS846 and sPIIANP, the remaining components of the model were almost identical to the original model. Since our goal was to determine the underlying domains in our complete dataset the first (five-component) model was considered our primary model. Nevertheless, the secondary model showed that sCS846 and sPIIANP were not so closely associated with the remaining variables. When variables in the primary analysis were categorized per component on the basis of maximum loading factors, components were as follows: Component 1 – uCTX-I, uNTX-I, sPINP, sOC, and uCTX-II, designated 'bone-CTX-II' cluster, component 2 – ESR, pLeptin, and hsCRP, designated 'inflammation' cluster, component 3 – sCOMP, sPIIINP, and sHA, designated 'synovium' cluster, component 4 – sC12C, pResistin, and pAdiponectin, designated 'C1,2C-adipokines' cluster, and component 5 – sPIIANP, sCS846, designated 'cartilage synthesis' cluster. Correlations among components were only very weak (maximum Pearson's correlation coefficient = 0.154, data not shown).

To determine whether these mutual associations between variables were dependent of radiographic OA status, the principal component analysis was repeated in participants with and without radiographic knee and/or hip OA separately (K&L grade ≥1 for one or more joints and K&L = 0 for all joints, respectively). The component structure in participants without radiographic OA was almost identical to that in the whole cohort (Tables SIII and SIV). However, in participants with radiographic OA some small differences were observed (Table IIIB and online supplementary data). This time, six components had 'eigenvalues' ≥1. uCTX-II still loaded to a comparable extent (loading factor 0.494) on the first component of bone markers, but this time loaded slightly more (loading factor −0.566) on the fifth component together with sPIIANP. Furthermore, sHA loaded on the sixth component together with sCS846. These fifth and six components had 'eigenvalues' of only 1.094 and 1.027, respectively, and the point of inflexion of the scree plot suggested four components as the most optimal model. When

Table IIIA
Principal component analysis of biomarkers in all subjects, irrespective of radiographic status (IIIA), and in subjects with radiographic OA only (IIIB). Structure matrix showing factors with 'eigenvalues' >1 as obtained after Direct Oblimin oblique rotation. Loading factors of biomarkers that were categorized per component are depicted in bold, while other loading factors >0.300 in the component concerned are depicted in italic. The communalities and scree plot are available in the online supplementary data

	1	2	3	4	5
uCTX-I	**0.905**	0.045	0.027	0.089	−0.063
uNTX-I	**0.861**	0.102	−0.004	0.103	−0.113
sPINP	**0.837**	−0.048	0.269	0.007	0.011
sOC	**0.793**	−0.118	0.204	0.131	0.020
uCTX-II	**0.547**	*0.342*	*0.355*	0.116	−0.213
ESR	0.059	**0.744**	0.054	0.159	0.020
pLeptin	0.043	**0.725**	0.105	−0.138	0.107
hsCRP	−0.192	**0.722**	0.041	*−0.377*	0.123
sCOMP	0.108	−0.090	**0.711**	0.050	0.035
sPIIINP	0.138	0.185	**0.685**	−0.224	−0.046
sHA	0.077	0.138	**0.653**	0.195	0.093
sC12C	0.062	0.112	0.088	**0.672**	0.037
pResistin	0.074	0.266	0.033	**−0.570**	0.058
pAdiponectin	*0.358*	−0.074	−0.001	**0.526**	−0.002
sPIIANP	−0.022	0.178	−0.049	−0.021	**0.759**
sCS846	−0.021	−0.016	0.116	0.015	**0.678**
'Eigenvalues'	3.406	1.927	1.681	1.381	1.143
% Of variance	21.7	12.8	8.8	7.5	6.7
Cronbach's alphas	0.842	0.619	0.327	0.252	0.173

Table IIIB

	1	2	3	4	5	6
uCTX-I	**0.908**	0.077	0.008	0.057	−0.139	−0.012
uNTX-I	**0.854**	0.149	−0.024	0.074	−0.191	−0.030
sPINP	**0.847**	−0.047	*0.309*	0.055	0.037	−0.042
sOC	**0.793**	−0.084	0.237	0.218	0.051	−0.117
pLeptin	0.073	**0.779**	0.075	−0.078	0.063	0.090
ESR	0.067	**0.761**	0.065	0.148	−0.031	−0.022
hsCRP	−0.166	**0.719**	0.013	*−0.456*	0.096	0.104
sCOMP	0.088	−0.041	**0.784**	0.154	0.092	−0.020
sPIIINP	0.144	0.153	**0.698**	−0.139	−0.143	0.073
sC12C	0.059	0.029	0.082	**0.647**	0.061	0.048
pAdiponectin	*0.316*	0.008	0.053	**0.605**	0.055	−0.217
pResistin	0.147	0.222	0.135	**−0.540**	0.156	−0.205
sPIIANP	−0.015	0.180	−0.002	0.011	**0.829**	0.131
uCTX-II	*0.494*	*0.345*	0.213	0.016	**−0.566**	0.193
sCS846	−0.008	0.023	−0.019	0.019	0.124	**0.822**
sHA	0.008	0.140	*0.486*	0.056	−0.248	**0.552**
'Eigenvalues'	3.427	2.080	1.444	1.230	1.094	1.027
% Of variance	21.4	13.0	9.0	7.7	6.8	6.4
Cronbach's alphas	0.832	0.654	0.364	0.218	0.278	0.237

a (forced) four-component model was investigated, sCS846 from the sixth component expectedly showed a low communality (0.132) and was excluded. uCTX-II, as before, clustered with the bone markers. sHA clustered with pLeptin, hsCRP, and ESR (Table SV).

Associations between demographics and biomarker levels are tabulated in Tables IV and V. Especially pLeptin, ESR, uCTX-I, and uNTX-I showed higher levels in women as compared to men (Table IV, left). Age was strongly associated with sHA and uCTX-II levels. BMI appeared to be strongly associated with pLeptin levels. Furthermore, postmenopausal women showed higher levels of all bone markers, uCTX-II, and sCOMP than premenopausal women (Table V, left). sCS846, by contrast, did not show any association with demographics. Statistically significant modification of the association of age and BMI with biomarker levels by gender (Table IV, middle) and/or menopausal status (Table V, middle) was mostly found for the biomarkers of bone metabolism. Especially associations of bone marker and uCTX-II levels with age differed between genders and between premenopausal and postmenopausal women: men and postmenopausal women showed no (or borderline significant) associations between age and biomarker levels, while premenopausal women showed positive associations (Tables IV and V, right).

Discussion

The current biomarker dataset in subjects with early symptomatic knee and/or hip OA is the largest in its kind and is especially valuable for its focus on early rather than late-stage disease.

The majority of biomarkers showed relevant associations with demographics, which were expectedly different between genders and/or menopausal status for some. Increased bone marker levels in postmenopausal women as compared to premenopausal women and men have been reported before[19–23]. In our study, bone markers were not associated with age in postmenopausal women but positively associated with age in our premenopausal women aged >45 years. This is in accordance with data from other authors demonstrating increasing bone marker levels during the age interval 45–60 years and stable plateau levels thereafter[20,22,24]. Men, by contrast, did not show associations between bone markers and age. Indeed, stable bone marker levels have been reported for men aged 45–65 years[23–25]. Also the negative associations between all biomarkers of bone metabolism and BMI are in accordance with literature on healthy subjects[20,26–29].

In our study, sCS846 did not show associations with demographics, as was also observed by Conrozier *et al.* in 56 hip OA patients[30]. Possibly, this absence of associations with demographics in our study may also be attributable to the observed low precision (i.e. high CV%) and concentration gradients within assay plates. Our data on associations between demographics and uCTX-II are in accordance with a study by Mouritzen *et al.* in healthy subjects showing increasing uCTX-II levels in women aged 50–55 years followed by a stable plateau phase, higher uCTX-II levels in postmenopausal women as compared to premenopausal women matched for age and BMI, and positive associations between uCTX-II and BMI[24]. Also our data on sCOMP were comparable to data from Jordan *et al.* on sCOMP levels in 769 randomly selected subjects from the Johnston County Osteoarthritis Project[31]. They demonstrated sCOMP levels to be positively associated with age and BMI and lower sCOMP levels in women than in men.

Principal component analysis enabled identification of clusters of interrelated biomarkers within the biomarker spectrum. The first, 'bone-CTX-II', cluster not only contained bone markers but also uCTX-II, which is supposed to be a marker of collagen type II degradation, and with that of cartilage matrix degradation. This observation does not stand on its own[32], since also other authors have observed associations between uCTX-II and bone markers[33–35] and Garnero *et al.* showed CTX-I, PINP and CTX-II loading onto one cluster in the ECHODIAH cohort of hip OA patients[36]. Accordingly, uCTX-II has been suggested to be primarily derived from osteoclastic resorption of calcified cartilage[37]. Interesting in this respect may be the localization of CTX-II epitope release at the cartilage–bone interface[38]. Using uCTX-II as a marker of (solely) cartilage degradation in clinical studies may be misleading, especially in trials of agents that influence bone metabolism. uCTX-II does not only reflect bone metabolism as is evidenced by the fact that uCTX-II also loaded to a considerable extent onto other components. Apparently, uCTX-II levels are associated with inflammation, synovial metabolism, and cartilage metabolism in addition to bone metabolism. uCTX-II was more strongly, inversely, associated with PIIANP in participants with radiographic signs of OA, which may reflect the uncoupling between cartilage synthesis and degradation that is presumed to be present in OA patients[39].

The second, 'inflammation', cluster contained pLeptin, hsCRP, and ESR, all associated with (systemic) inflammation and cardiovascular disease/metabolic syndrome[40]. This cluster did not show so many associations with biomarkers of joint metabolism, except for uCTX-II (mentioned above) and sHA in the four-component model in subjects with radiographic OA. The latter may reflect the synovitis that may be present to a higher extent in these subjects.

The third, 'synovium', cluster contained biomarkers that have been related to the low-grade synovitis that is present in OA: sPIIINP[41], sHA[42], and sCOMP. Again, Garnero *et al.* showed exactly the same component in the ECHODIAH cohort[36]. sCOMP has been demonstrated to be present in synovial tissue, to be produced by synoviocytes[43], and associated with clinically[44,45] and ultrasonographically diagnosed[42] synovitis and/or effusion in OA. sCOMP levels should not only be interpreted in the context of cartilage degradation[46], but should also be interpreted in the context of synovitis. Bone synthesis markers showed some positive associations with this cluster, possibly reflecting a link between osteophytosis and synovitis[42].

The fourth, 'C1,2C-adipokines', cluster contained sC1,2C, pResistin, and pAdiponectin. Literature on adipokines and C1,2C is scarce. One small study showed increased C1,2C levels upon adiponectin stimulation of *ex vivo* OA cartilage explants[7], which is exactly opposite our findings. This and the third, 'inflammation', cluster did not show so many associations with biomarkers of joint metabolism, arguing against major involvement of adipokines in OA.

Table IV
Associations between demographic variables and biomarkers for all participants and for genders separately when interaction terms with gender (men as reference) were statistically significant. Associations were investigated through multiple linear regression analyses with in each model one of the biomarkers as dependent variable and in all models demographic variables as independent variables. Statistically significant results are bold ($P < 0.200$ for interaction terms)

	Gender		Age		BMI		Interaction terms		Male				Female			
							Gender*age	Gender*BMI	Age		BMI		Age		BMI	
	Stand beta	*P*	Stand beta	*P*	Stand beta	*P*	*P*	*P*	Stand beta	*P*	Stand beta	*P*	Stand beta	*P*	Stand beta	*P*
Cartilage																
uCTX-II	0.191	**<0.001**	0.242	**<0.001**	0.112	**<0.001**	**0.029**	0.492	0.159	**0.023**			0.267	**<0.001**		
sCOMP	−0.157	**<0.001**	0.184	**<0.001**	0.173	**<0.001**	0.353	0.622								
sPIIANP	0.059	0.070	0.075	**0.020**	0.099	**0.002**	0.918	0.316								
sCS846	0.000	0.989	−0.031	0.339	0.061	0.063	**0.167**	0.533	−0.132	0.064			−0.006	0.871		
Cartilage/bone																
sC1,2C	0.125	**<0.001**	0.030	0.349	−0.093	0.004	0.699	0.847								
Bone																
uCTX-I	0.278	**<0.001**	0.160	**<0.001**	−0.137	**<0.001**	**0.001**	0.422	−0.021	0.767			0.211	**<0.001**		
uNTX-I	0.291	**<0.001**	0.183	**<0.001**	−0.111	**<0.001**	**0.025**	0.472	0.073	0.304			0.221	**<0.001**		
sPINP	0.173	**<0.001**	0.124	**<0.001**	−0.100	**0.002**	**0.006**	0.264	−0.047	0.513			0.165	**<0.001**		
sOC	0.130	**<0.001**	0.170	**<0.001**	−0.176	**<0.001**	**0.001**	0.345	−0.019	0.785			0.212	**<0.001**		
Synovium																
sHA	−0.084	**0.005**	0.367	**<0.001**	0.117	**<0.001**	**0.014**	0.442	0.299	**<0.001**			0.388	**<0.001**		
sPIIINP	−0.027	0.390	0.034	0.284	0.229	**<0.001**	0.533	**0.062**			0.318	**<0.001**			0.208	**<0.001**
Adipokines																
pAdiponectin	0.320	**<0.001**	0.149	**<0.001**	−0.243	**<0.001**	0.211	**0.113**			−0.156	**0.026**			−0.276	**<0.001**
pLeptin	0.548	**<0.001**	0.010	0.585	0.596	**<0.001**	0.905	0.923								
pResistin	0.041	0.204	−0.025	0.427	0.160	**<0.001**	0.216	0.426								
Inflammation																
hsCRP	0.074	**0.013**	0.038	0.200	0.393	**<0.001**	0.945	0.711								
ESR	0.318	**<0.001**	0.106	**0.001**	0.173	**<0.001**	0.433	**0.023**			0.017	0.811			0.220	**<0.001**

Table V
Associations between demographic variables and biomarkers for women and for premenopausal and postmenopausal women separately when interaction terms with menopausal status (premenopausal women as reference) were statistically significant. Associations were investigated through multiple linear regression analyses with in each model one of the biomarkers as dependent variable and in all models demographic variables as independent variables. Statistically significant results are bold ($P < 0.200$ for interaction terms)

	Menopausal status		Age		BMI		Interaction terms		Premenopausal women				Postmenopausal women			
							Menop*age	Menop*BMI	Age		BMI		Age		BMI	
	Stand beta	*P*	Stand beta	*P*	Stand beta	*P*	*P*	*P*	Stand beta	*P*	Stand beta	*P*	Stand beta	*P*	Stand beta	*P*
Cartilage																
uCTX-II	0.137	**0.008**	0.188	**<0.001**	0.084	**0.035**	**0.003**	0.678	0.368	**<0.001**			0.096	**0.041**		
sCOMP	0.122	**0.022**	0.062	0.241	0.141	**0.001**	**0.010**	0.890	0.245	**0.004**			0.000	0.998		
sPIIANP	0.031	0.559	0.051	0.339	0.099	**0.016**	0.701	0.828								
sCS846	0.033	0.535	0.015	0.775	0.065	0.119	0.866	0.922								
Cartilage-bone																
sC1,2C	−0.002	0.970	0.018	0.740	−0.120	**0.004**	**0.069**	0.400	0.160	0.067			−0.022	0.644		
Bone																
uCTX-I	0.239	**<0.001**	0.051	0.323	−0.179	**<0.001**	**<0.001**	**0.023**	0.371	**<0.001**	−0.015	0.853	−0.042	0.366	−0.238	**<0.001**
uNTX-I	0.207	**<0.001**	0.079	0.127	−0.147	**<0.001**	**<0.001**	**0.057**	0.332	**<0.001**	−0.008	0.927	−0.008	0.927	−0.197	**<0.001**
sPINP	0.240	**<0.001**	−0.020	0.696	−0.164	**<0.001**	**<0.001**	**0.006**	0.276	**0.001**	0.037	0.662	−0.092	**0.045**	−0.231	**<0.001**
sOC	0.256	**<0.001**	0.022	0.662	−0.239	**<0.001**	**<0.001**	**0.048**	0.323	**<0.001**	−0.093	0.264	−0.064	0.156	−0.299	**<0.001**
Synovium																
sHA	0.075	0.130	0.333	**<0.001**	0.108	**0.005**	0.668	0.384								
sPIIINP	0.065	0.219	−0.017	0.754	0.180	**<0.001**	0.596	0.664								
Adipokines																
pAdiponectin	0.050	0.326	0.065	0.203	−0.296	**<0.001**	**0.114**	0.512	0.232	**0.006**			0.010	0.829		
pLeptin	0.014	0.700	0.006	0.871	0.735	**<0.001**	0.874	0.349								
pResistin	−0.092	0.080	−0.009	0.868	0.177	**<0.001**	0.412	**0.155**			−0.033	0.704			0.227	**<0.001**
Inflammation																
hsCRP	−0.055	0.264	0.085	0.083	0.426	**<0.001**	0.486	0.415								
BSE	0.028	0.597	0.089	0.092	0.197	**<0.001**	0.781	0.528								

The fifth, 'cartilage synthesis', cluster contained sPIIANP and sCS846. Both are indicative of synthesis of cartilage matrix, sPIIANP representing collagen synthesis[38,39] and sCS846 representing glycosaminoglycan synthesis[47]. Accordingly, Otterness *et al.* showed CS846 and C-propeptide of type II collagen (associated with type II collagen synthesis) loading onto one component[48]. We do not have a clear explanation for the association between sHA and sCS846 that was specifically observed in the sixth cluster in subjects with radiographic OA. Possibly, in these subjects synovitis has some association with cartilage synthesis, although we would then rather expect sHA and sCS846 loading inversely onto this cluster.

Interpretation of the, mostly rather low, Cronbach's alphas of the identified clusters is not so straightforward. Although they might indicate that biomarkers in the clusters concerned do not represent one underlying domain, which may be true, they probably also represent the variable metabolism and kinetics between biomarkers.

Concluding, the observed associations between biomarkers and demographics as well as the identified biomarker clusters extended on literature on individual biomarkers and our own expertise. This was interpreted as evidence for validity of our biomarker dataset. Also, some interesting associations were observed that warrant more cautious interpretation of some biomarkers than is normally done in current biomarker literature.

Obvious strengths of the current study are its size, the multiple biomarkers and adipokines that were assessed simultaneously, and the small numbers of missing biomarker data. Of course, this study has limitations also. First of all, biomarkers were only assessed in baseline samples. Serial biomarker assessments in this longitudinal cohort would also be very valuable and may be performed in the future. Secondly, a healthy control group would have been interesting to compare (associations between) biomarkers between healthy and OA subjects, but was not included in our study. Thirdly, a major limitation of biomarkers in general is the uncertainty about the biological process(es) they reflect. Furthermore, associations between systemic biomarker levels and joint metabolism, if any, are potentially obscured by factors such as variable biomarker release from joints due to physical activity[49] and extra-articular metabolism[50]. Finally, it should be emphasized that further studies would be needed to confirm the component structure that was found in our biomarker dataset. Especially interesting in this perspective will be the biomarker assessment that is planned in the Osteoarthritis Initiative (OAI).

Altogether we believe that we have performed valid assessment of a wide spectrum of biomarkers in CHECK, a 10-year prospective cohort of 1,002 subjects with (very) early symptomatic OA. These data will prove invaluable for future analyses of cross-sectional and predictive relations between biomarkers and structural and clinical joint parameters in early knee and hip OA.

Author contributions

WvS, FL, NJ, JdG and JB have made substantial contributions to the conception and design of the study, obtaining of funding, and acquisition of data. WvS, FL, and PW were primarily involved in the analysis and interpretation of data. WvS wrote article drafts that were critically revised for important intellectual content by all authors. All authors gave their approval of the final version to be submitted. WvS and FL take responsibility for the integrity of the work as a whole, from inception to finished article (w.e.vanspil@umcutrecht.nl; f.lafeber@umcutrecht.nl).

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Competing interests

There are no competing interests to be declared by any of the authors.

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.joca.2012.04.004.

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