Contents lists available at ScienceDirect

Journal of Clinical & Translational Endocrinology

Original research

Crohn's disease patient serum changes protein expression in a human mesenchymal stem cell model in a linear relationship to patients' disease stage and to bone mineral density☆

Martina Blaschke[a,*], Regine Koepp[a], Christof Lenz[c,d], Jochen Kruppa[e,f], Klaus Jung[e,g], Heide Siggelkow[a,b]

[a] Clinic of Gastroenterology and Gastrointestinal Oncology, University Medical Center Göttingen, 37075 Göttingen, Germany
[b] MVZ Endokrinologikum Göttingen, von Siebold-Str. 3, 37075 Göttingen, Germany
[c] Bioanalytical Mass Spectrometry, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany
[d] Institute of Clinical Chemistry, University Medical Center, 37075 Göttingen, Germany
[e] Department of Medical Statistics, University Medical Center Göttingen, Humboldtallee 32, 37073 Göttingen, Germany
[f] Genomics and Bioinformatics of Infectious Diseases, University of Veterinary Medicine Hannover, Institute of animal breeding and Genetics, 30559 Hannover, Germany
[g] Institute of Biometry and Clinical Epidemiology, Charite, University Medical Center Berlin, Chariteplatz 1, 10117 Berlin, Germany

ABSTRACT

Background: Crohn's disease (CD) is associated with a higher prevalence of osteoporosis, a complication that is recognized as a significant cause of morbidity. Its pathogenesis is controversial, but the activity of CD is one contributing factor.
Methods: We stimulated SCP-1 cells (mesenchymal stem cell line) under osteogenic conditions with serum from adult patients with CD in the symptomatic phase (SP) and in remission (R) and with control sera. Concentrations of IL-6, IL-1 beta, and TNF alpha in the sera were measured. Patients were classified as normal or osteopenic/ osteoporotic based on bone mineral density (BMD) T-score measurements. After 14 days in culture, protein expression and gene ontology (GO) annotation analysis was performed.
Results: Cytokine concentrations (IL-6, IL-1 beta, TNF alpha) varied within sera groups. None of the cytokines were significantly increased in the symptomatic phase compared to remission. Protein analysis revealed 17 proteins regulated by the SP versus R phase sera of disease. A linear relationship between CDAI (Crohn's disease activity index) and normalized protein expression of APOA1 and 2, TTR, CDKAL1 and TUBB6 could be determined. Eleven proteins were found to be differentially regulated comparing osteoporosis-positive and osteoporosis-negative sera. Gene annotation and further analysis identified these genes as part of heme and erythrocyte metabolism, as well as involved in hypoxia and in endocytosis. A significant linear relationship between bone mineral density and normalized protein expression could be determined for proteins FABP3 and TTR.
Conclusion: Our explorative results confirm our hypothesis that factors in serum from patients with CD change the protein expression pattern of human immortalized osteoblast like cells. We suggest, that these short time changes indeed influence factors of bone metabolism.

Introduction

Crohn's disease (CD) is a complex multifactorial disease, which is associated with extra intestinal manifestations. One of the organs affected is the skeleton [1]. Therefore, it is important to address the mechanisms of osteoporosis in patients with CD to prevent and treat bone loss [2]. Osteoporotic fractures have a negative impact not only on

the quality of life but also on life expectancy [3–5]. CD patients have a 1.2 to 1.7-fold increased relative risk of vertebral and hip fractures, leading to both direct and indirect costs [6–9]. Predicting which individuals have a higher risk remains controversial, yet hypogonadism, less physical activity, malnutrition, malabsorption, vitamin D deficiency or inactivity, disease activity, and glucocorticoid use, in particular, are thought to be the main contributing factors. However, bone

disease may also be present without glucocorticoid treatment [1,10–12,13,14]; therefore, the effect of the disease itself has to be taken into account. Cytokines produced by inflamed intestine in CD may affect bone and hence contribute to bone loss in patients with CD [15]. During the different inflammatory phases in CD cytokines from the gut are secreted into the serum. IL-1 beta, TNF alpha, and especially IL-6 are most likely the main contributing factors to bone deterioration [16]. IL-6 has been identified in sera of children to be responsible for bone loss by decreasing osteoblast differentiation [17]. Interestingly, using serum from children with CD on bone organ cultures only bone formation was decreased and osteoclast activity was unchanged [18]. In newly diagnosed children with CD biochemical bone turnover markers indicated reduced bone formation and also reduced bone resorption [17]. Other studies concerning adult bone have identified IL-1 beta, TNF alpha, and IL-6 to be increased in patients with CD or colitis ulcerosa [15,16,19]. We were able to show with serum from adult patients with CD, that the cytokine combination of IL-1 beta, TNF alpha, and IL-6, but not the individual cytokines of identical concentration, influence bone formation and bone resorption [20]. If bone resorption is induced it seems to be an effect of receptor activator of NF-κB ligand (RANKL) induction, an osteoclast activating soluble ligand produced by osteoblasts and osteocytes, a potent inducer of osteoclastogenesis and activity [15].

Interestingly, not all patients with CD develop osteoporosis. Either the individual bone characteristics or the serum components in these patients may affect or not affect bone health.

We therefore hypothesized here that factors in the serum of patients with CD decisively influence bone turnover. We investigated the effect of serum from patients with CD and healthy individuals on immortalized human osteoblast-like cells. The patient serum was grouped according to different phases of the disease and to bone mineral density (BMD) parameters.

Material and methods

Symptomatic phase and remission sera sampling

We recruited patients with active disease symptoms with CD either at the emergency clinic or at the Clinic for Gastroenterology and Gastrointestinal Oncology (both University Medical Center Goettingen). CD diagnosis was based on endoscopic, histological, or radiological findings. The symptomatic phase of CD was identified using the Crohn's disease activity index (CDAI; supplementary data Table a) [21], in which a score greater than 150 was defined as symptomatic disease. From 23 patients screened during two years only 7 fulfilled the stringent criteria (see below). In another study investigating steroid-free CD patients with active or symptomatic disease, the authors were able to include 99 patients from 34 centers, therefore only around 3 for each center, showing the difficulty of this inclusion criterion [11]. Hence glucocorticoids decisively influence bone metabolism and bone turnover returned to normal in patients not before 4 weeks after cessation of glucocorticoid therapy [22]. Therefore, patients were not included if they received treatment with any steroid or immunosuppressive compound one months prior to analysis in symptomatic disease and in remission. The use of calcium and vitamin D supplements or estrogen was allowed (patient's characteristics please see supplementary data Tables b and c).

Upon inclusion in the study, routine blood analysis was performed. An additional 50 ml blood was drawn, placed directly on ice, and centrifuged within minutes; the resulting serum was batched and stored at −70 °C. When the same patient reached remission and returned for routine examination, another 50 ml blood was drawn, and serum was batched and stored at −70 °C. Steroid treatment had to be finished at least 4 weeks before blood sampling. This study was approved by the Ethics Committee of the University Medical Center Goettingen, and informed consent was signed by all subjects. The characteristics of these

Table 1
Parameter presenting data (mean values ± standard deviation) comparing osteoporosis positive and osteoporosis negative patients.

Parameter	**Ost +** [c]	**Ost −** [d]	P-value	Total
Sex (male/female)	1/2	2/1		6
T-score femoral neck	**−1.9 ± 0.4**	**0.3 ± 0.97**	**< 0.05**	
T-score spine	**−2.1 ± 0.7**	**0.3 ± 1.15**	**< 0.05**	
IL-6 (pg/mL) SP[a]	15.16 ± 10.73	9.8 ± 15.53	ns	12.48 ± 12.3
IL-6 (pg/mL) R[b]	8.41 ± 8.03	10.20 ± 15.75	ns	9.3 ± 11.22
P-value	ns	ns		
TNF α (pg/mL) SP	5.37 ± 4.66	1.84 ± 3.17	ns	3.61 ± 4.05
TNF α (pg/mL) R	56.17 ± 87.33[*]	6.17 ± 10.68	ns	31.17 ± 62.01
P-value	ns	ns		
IL-1 β (pg/mL) SP	0.37 ± 0.078	1.0 ± 1.61	ns	0.68 ± 1.08
IL-1 β (pg/mL) R	0.13 ± 0.16	1.34 ± 2.26	ns	0.74 ± 1.58
P-value	ns	ns		
CDAI[e] SP	**349 ± 82**	284 ± 61		**316 ± 73**
CDAI R	**104 ± 71**	91 ± 126		**98 ± 92**
P-value	**< 0.02**	ns		**< 0.001**

Numbers in **bold** are significant values.
[a] SP: symptomatic phase.
[b] R: remission.
[c] Ost +: osteoporosis positive.
[d] Ost −: osteoporosis negative.
[e] CDAI: crohn's disease activity index.
[*] 1 patient was treated with infliximab in between phases, 3 months prior sampling and showed extremely high TNF alpha values.

patients have been published previously [13]. Briefly, patients were grouped according to their BMD results based on WHO criteria, using a T-score below −1 at the femoral neck or spine to assign patients to the osteopenia/osteoporosis group (Table 1). In addition, sera from healthy individuals (non-CD; non-osteoporotic) were batched and treated like the CD-patient sera.

Bone densitometry

Bone densitometry using dual X-ray absorptiometry (DXA) was performed at the lumbar spine (L1-L4) and the left femoral neck in all patients with CD during the first week of their stay in the hospital. Osteoporosis was diagnosed when the BMD value was 2.5 or more standard deviations below the mean of a young reference population (T-score), and osteopenia was diagnosed as a T-score below −1. For comparison, the control population data provided by the DXA manufacturer (Hologic, QDR 1000) was used.

Cytokine measurement

Serum cytokine levels were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's specifications (Table 1). Kits for IL-6 (reference values: 0.447–9.96 pg/ml, minimal detectable level: 0.016–0.110 pg/ml), and IL-1 beta (reference values: < 1.996 pg/ml, minimal detectable level: < 0.1 pg/ml) were purchased from R&D Systems (Minneapolis, USA). For TNF alpha (DPC Biermann GmbH, Bad Nauheim, Germany), the reference values from the manufacturer were < 8.1 pg/ml and minimal detectable level was 0.1 pg/ml. Intra-assay precision was 2.6–3.6%, and inter-assay precision was 4–6.5%.

Cell culture

Disposable products for cell culture were obtained from Nunc (Roskilde, Denmark) and cell culture media and fetal calf serum (FCS)

were from PAA (Cölbe, Germany); the medium supplements (antibiotics and glutamine) were purchased from GIBCO-BRL (Eggenstein, Germany). All other reagents were purchased from Sigma Chemical Co. (Munich, Germany). Primers for reverse transcriptase polymerase chain reaction (RT-PCR) were obtained from Invitrogen (Carlsbad, CA, USA).

SCP-1 ([23]; single-cell-picked clone of hTERT immortalized human mesenchymal stem cells) cells were split 1:5 every week and cultured under standard conditions (10% FCS in MEM-alpha) for cell culture maintenance. Cells were trypsinized for experiments and plated on 6-well dishes at a density of 5×10^4 cells/ml. At confluence, cell media was supplemented with 10 mM beta-glycerophosphate (bGP) and 10 µM ascorbic acid 2-phosphate (ascP) for basic osteogenic differentiation in addition to stimulation with 1% human patient serum every 3 days. The use of 1% serum was successfully tested and compared to use of 10% serum regarding cell viability and differentiation. According to the findings of Sylvester [17] we decided to use 1% serum. Sera applied were received from 6 different CD patients and one healthy control male individual. Patients were either in symptomatic phase (SP) or in remission (R) at the time point of sampling. The remission phase sera were drawn from the identical patient as the symptomatic phase sera, but after a mean time of 8 month later. Protein was isolated from SCP-1 cells after 14 days of continuous treatment with sera. The healthy control serum was treated as the patient sera and applied to the SCP-1 cell culture pure or plus an addition of a cytokine combination (supplementary data Fig. A). The composition of cytokine combination (10 pg/mL IL-6, 1 pg/mL IL1β, and 5 pg/mL TNFα) was calculated on the basis of mean cytokines concentrations measured in the sera of CD patients [13,20,24]. The combination of cytokines will be referred to as 'plus cytokines' in the following.

Protein isolation

For protein isolation, cells were washed twice with phosphate buffered saline (PBS) and afterwards treated with hot protein lysis buffer containing 10% (v/v) glycerol, 1% (v/v) Triton x 100, and 1 mM EGTA in 50 mM HEPES (pH 7.5). After homogenization of cells through a syringe, the material was kept on ice, followed by centrifugation at 14,000 rpm at 4 °C. Protein concentration was detected in supernatant by using the BCA method (Pierce, Waltham, USA). Proteins were purified by precipitation using a standard acetone precipitation protocol (acetone: sample 4:1, v/v, −20 °C, overnight). Protein preparations were dissolved using sodium 3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl) methoxy]-1-propanesulfonate (RapiGest, Waters, Milford, USA) cleavable surfactant [25]. After reduction and alkylation of cysteine residues with dithiothreitol and iodoacetamide, proteins were digested using sequencing grade porcine trypsin (Promega, Madison, USA) at a 1:50 enzyme-to-substrate ratio (w:w). Following acidic cleavage of the surfactant, the resulting fatty acids were pelleted and removed by centrifugation. The resulting peptide mixtures were dried in a SpeedVac centrifuge and stored at −20 °C prior to analysis.

LC/MS/MS acquisition

Protein digests were analyzed on a nanoflow chromatography system (Eksigent nanoLC425) hyphenated to a hybrid triple quadrupole-time of flight mass spectrometer (TripleTOF 5600+) equipped with a Nanospray III ion source (Ionspray Voltage 2200 V, Interface Heater Temperature 150 °C, Sheath Gas Setting 10) and controlled by Analyst TF 1.6 software build 6211 (all AB Sciex, Darmstadt, Germany). In brief, peptides from each digest were dissolved in 160 µl loading buffer (2% aqueous acetonitrile vs. 0.1% formic acid). For each analysis, 1 µg protein equivalent was concentrated and desalted on a trap column (Waters Synergy C18 5 µm, 20 × 0.180 mm, 160 µl loading buffer) and separated by reversed phase-C18 nanoflow chromatography (Waters TSS-3 1.8 µm, 250 × 0.075 mm, linear gradient 90 min 5–35% acetonitrile vs. 0.1% formic acid, 300 nl/min, 50 °C).

Qualitative LC/MS/MS analysis was performed using a Top25 data-dependent acquisition method with an MS survey scan of m/z 380–1250 accumulated for 250 ms at a resolution of 35.000 FWHM. MS/MS scans of m/z 180–1750 were accumulated for 100 ms at a resolution of 17.500 FWHM and a precursor isolation width of 0.7 FWHM, resulting in a total cycle time of 3.4 s. Precursors above a threshold MS intensity of 200 cps with charge states 2+, 3+, and 4+ were selected for MS/MS, and the dynamic exclusion time was set to 15 s. One technical replicate of each sample was acquired for qualitative analysis for protein identification and generation of a spectral library for targeted data extraction.

For SWATH analysis, MS/MS data were acquired for 34 precursor segments of 25 m/z each, resulting in a precursor m/z range of 400–1250. Fragments were produced using Rolling Collision Energy Settings and fragments acquired over an m/z range of 380–1600 for an accumulation time of 110 ms per segment. Including a 250 ms survey scan, this resulted in an overall cycle time of 4.0 s. Three technical replicates of each sample were acquired for quantitative analysis.

LC/MS/MS data processing

Protein identification was achieved using ProteinPilot Software version 5.0 build 4304 (AB Sciex) at "thorough" settings. A total of 386.031 MS/MS spectra from the combined qualitative analyses were searched against the *Homo sapiens* reference proteome from UniProtKB (revision 10-2014, 88717 protein entries). Global false discovery rates (FDR) were adjusted to 1% at both the protein and peptide level using a forward/reverse decoy database approach.

SWATH peak extraction was achieved in PeakView Software version 2.1 build 11041 (AB Sciex) using the SWATH quantitation microApp version 2.0 build 2003. Peak areas were extracted for the ten highest scoring peptides per protein group at 6 transitions per peptide, with an extracting ion current (XIC) width of 75 ppm and an XIC window of 14 min, and filtered to an estimated FDR of 1% [26]. The resulting peak areas were then exported at the fragment, peptide, and protein level for further statistical analysis.

Protein analysis

Raw expression levels were first normalized over all samples using the quantile method and then log2-transformed for variance stabilization. Hierarchical cluster analysis with single linkage was performed to identify potential outliers and with complete linkage to explore potential subgroups. Furthermore, the presence of clusters with regard to the experimental factors (phase and BMD T-score) was explored by principal component analysis.

Differential analysis between the levels of each experimental factor was carried out by linear models for microarray experiments, including the information for the technical replicates [27]. Raw p-values for the differential expression were adjusted using the method described by Benjamini and Hochberg [28] to reduce the number of false positives and to control for a false discovery rate of 5%. Fold changes were reported on the log2-scale, i.e., a log2-fold change of 1 reflects a twofold up-regulation and a log2-fold change of −1 reflects a twofold down-regulation. Confidence intervals for the fold changes were calculated according to the method described by Jung et al. [29].

Significantly up- or downregulated proteins were further studied for enrichment of specific gene ontology (GO) terms by Fisher's exact test [30]. Resulting raw p-values were again adjusted to control for a false discovery rate of 5% (Swiss-Prot reviewed (47,810 entries) protein-gene matching).

All analyses were carried out using the software R (www.r-project.org). Specifically, the differential analysis was performed using the R-package 'limma'.

GO analysis

Gene set enrichment for gene ontology biological process annotation was analyzed using DAVID Bioinformatics Resources 6.7, NIAID/NIH (https://david.ncifcrf.gov) to identify the most significant cellular processes affected by sera from patients with CD [31]. As proteins background set all human proteins were used. For data analysis the GOterm_BP_all (biological process_all) was consulted.

Further statistical analysis

The data were analyzed using Prism GraphPad 4 software (San Diego, USA). All experimental errors are presented as the standard deviation (SD). Statistical significance was calculated using a One way ANOVA followed by a Bonferroni analysis or unpaired *t*-test. Significance was set at $P < 0.05$.

Results

Patients

Analysis of patient T-scores revealed classification of 50% of patients as osteopenia/osteoporosis positive (Ost+), with the lowest T-Score value measured at the femoral neck or the spine ranging from -1.4 to -2.8 (T-score femoral neck: mean value: -1.9 ± 0.4; n = 3). T-score values ranging from -0.5 to $+1.4$ (T-score femoral neck: mean value: 0.3 ± 0.97; n = 3) were classified as osteoporosis negative (Ost-) (Table 1). Symptomatic phase and remission were classified by CDAI (supplementary data Tables a and b) [32].

Cytokine measurement in patient serum showed a high variance in IL-6 concentration between the patient sera in the symptomatic phase (0.4–27.7 pg/ml) as well as in remission. This led to no significant differences between IL and 6 concentrations. TNF alpha was increased in half of the patients during the symptomatic phase with values ranging from 5.5 to 8.4 pg/ml. All sera with increased TNF alpha levels were from female patients. No significant changes could be found between the symptomatic phase and remission sera, in part due to one extremely high TNF alpha value in remission. Interestingly, this patient was treated with Infliximab in between the CD stages, but 3 month prior to serum sampling. This IL-1 beta values ranged from 0.05 to 2.85 pg/ml during the symptomatic phase and no significant differences in IL-1 beta were observed between phases (Table 1). As published before – with in part overlapping patient serum samples – cytokine levels were more than 10fold higher in comparison to age-matched healthy controls for all three cytokines [13]. The disease duration in CD patients with osteoporosis/osteopenia (14 ± 3.1 years) was significantly longer than in those without osteoporosis (5 ± 3.6 years) and correlated significantly negative with BMD at the spine (supplementary data Fig. B and Table b1).

Verification of osteogenic lineage

To verify the osteogenic lineage of SCP-1 cells in 1% human serum, we applied real-time-PCR analysis of osteoblast-specific genes (ALPL, Col1A1, BGLAP (osteocalcin), Runx2, DKK1, TNFRSR11B (OPG), and TNFSF (RANKL)) for the osteoblastic lineage and also, as a negative control, a set of genes (FABP4 (aP2), LPL, PPARgamma) for the adipogenic lineage. Expression of osteogenic markers was similar to the expression observed when cells were incubated under equivalent standard conditions (10% FCS) (supplementary data Fig. C), indicating an osteogenic differentiation of the SCP-1 cells as has been shown before [33]. Expression of OC was on average 3-fold lower after incubation with 1% patient sera and Col1A1 was 10-fold higher after incubation in 1% patient sera. This constellation of bone markers indicated an early stage of osteoblastic differentiation prior to the stage of matrix formation [34–36].

Proteomics

Similar amounts of protein (and also RNA) were isolated from the cell cultures independent of serum type applied. Cell proliferation was not affected by different sera. No changes in phenotype were observed under microscopic control.

Proteome analysis revealed 1444 identified proteins. The following comparisons were performed to analyze if proteins are differentially expressed in SCP-1 cells and to identify proteins, which might be involved in the formation of osteoporosis (Supplementary data Fig. A experimental design).

a) Symptomatic phase sera versus remission sera (cutoff point was set to Padjust < 0.1 and fold changes > 1.6) and versus healthy control sera (explorative comparison) to analyze which proteins and pathways are induced during the symptomatic phase
b) Non-CD control sera versus non-CD control sera plus cytokine combination (explorative comparison) to estimate which effect on protein expression is caused by the increased cytokines as measured in CD-patients
c) CD sera during both phases in comparison to healthy control serum supplemented with cytokines to identify the effect of the CD specific sera (explorative comparison)
d) Osteoporosis-positive sera versus osteoporosis-negative sera (cutoff point was set to Padjust < 0.05 and fold changes > 1.6) and versus healthy control sera (explorative comparison).

All listed comparisons and identified proteins are listed in Table 2 (p < 0.05, foldchange < or > 1.6). All comparisons are described in detail in Section "Comparative analyses of proteome results".

Comparative analyses of proteome results
Comparison between symptomatic phase sera samples versus remission sera samples. Comparing incubation with symptomatic phase sera versus remission sera, 17 proteins were identified to be significantly regulated by symptomatic phase sera (Padjust < 0.1; Table 3). Five Proteins were more than 1.6fold decreased in SCP-1 cells incubated with symptomatic phase sera: APOA2 (apolipoprotein A2), APOA1 (apolipoprotein A1), TBB6 (tubulin beta-6-chain), TTR (transthyretin), CDKAL1 (CDK5 (cyclin dependent kinase) regulatory subunit associated protein 1 like 1) and two proteins were more than 1.6fold increased: KRT10 and KRT5 (keratin 10 and 5). The latter are keratins and are involved in the cytoskeleton pathway and epidermal structuring.

A graphical presentation of the changes in normalized protein expression of APOA1, APOA2, TUBB6, TTR, CDKAL1 and KRT10 in SCP-1 cells treated with symptomatic phase sera versus remission sera is given in Fig. 1.

The grouped presentation of normalized protein expression reflected a significant decrease in APOA1, APOA2, TTR, TUBB6 and CDKAL1 expression after stimulation with symptomatic phase sera compared to the remission phase confirming the results obtained by the statistical analysis. A correlation analysis of CDAI values determined during symptomatic and remission phase and the identified regulated proteins is presented in Fig. 2 and showed a significant linear relationship between the CDAI and the downregulated proteins tubulin beta-6 (TBB6; $r^2 = 0.56$), transthyretin (TTR; $r^2 = 0.48$), apolipoprotein A1 (APOA1; $r^2 = 0.50$) and apolipoprotein A2 (APOA2; $r^2 = 0.62$). For the remaining proteins cyclin dependent kinase (CDKAL1; $r^2 = 0.08$) and the KRT10 (KRT5, data not shown) no significant correlation between CDAI and protein expression could be obtained.

Our results show, that the activity of CD characterized by CDAI influences proteins in SCP-1 cells by the serum of the corresponding patients. In addition, the degree of activity represented by CDAI seems to vary this effect.

GO analysis of proteins, regulated by symptomatic phase versus remission

Table 2

Significantly affected SCP-1 proteins by CD patient sera.[1]

Sera applied to SCP-1 cells	Upregulated proteins*	Downregulated proteins*
a) CD symptomatic phase (SP) vs CD remission (R) +(Table 3)	KRT10 (Figs. 1–3), KRT5	APOA2, APOA1, TTR, TUBB6, CDKAL1 (Figs. 1–3)
b) Healthy control plus cytokines vs Healthy control (Table 4)	SOD2, CAMK2D (Fig. 4), TARDBP, RPL7	KRT1, KRT10 (Figs. 1–3), KRT5, SLC44A2 (Fig. 4), PTGIS, ARL3, H2AFX, TSPO, ACO1, PSMB10, PLEKHO2 (Fig. 4), RPS13, KRT6A(TUBB6), (TTR), (CDKAL) (Fig. 3)
c) CD vs Healthy control plus cytokines	SLC44A2 (Fig. 4), MAPK14, IFITM3, GBP1, EIF4A1, ARMT1, SSR4. BOLA2, TIGAR, PSMC1, GOLT1B, NPC1, TSPO, MAVS, LRRC47, GPX8, CTSL, HUWE1, MPST	GLUD1 (Fig. 4), CAMK2D (Fig. 4), RPL18, CAVIN1, RPL13, RPL10
d1) CD osteoporosis positive vs CD osteoporosis negative (Table 5)	FABP3 (Figs. 5 and 6)	HBB, HBA1/HBA2, JCHAIN, TTR, A2M, FTH1 (Figs. 5 and 6.)
d2) CD osteoporosis positive vs Healthy controls	FABP3 (Fig. 5)	HBB, HBA1/HBA2 (Fig. 5), GLUD1, KRT10, KRT1, S100A7
d3) CD osteoporosis negative vs Healthy controls	IGHG2, GBP1, EIF4A2, SLC44A2, APOD, BPNT1, LEPROT	GLUD1, CAVIN1, CASP14, RPL30, RPL19, KRT10, HTRA1, S100A7, CAD

+ Padjust was < 0.1 for SP versus R.

[1] Please see Figure A in supplementary material for comparative analyses. Low types a), b), c) d1–d3) correspond to the low types in Figure A (supplementary material).

* Listed proteins were significantly changed with a Padjust < 0.05 and with a fold change > 1.6.

Table 3

Proteins identified as regulated by symptomatic phase sera (R/SP; p < 0.1).

ProtID	P	Padjust	log2FC	Fold change up or down	GENES
P60709	8.85E−06	0.0128	0.32	1.25	ACTB
P13645	4.28E−05	0.0235	−1.29	*2.44*	*KRT10 KPP*
P02647	5.41E−05	0.0235	0.68	**1.61**	**APOA1**
C9JA28	9.22E−05	0.0235	0.37	1.29	SSR3
P05387	9.31E−05	0.0235	0.19	1.14	RPLP2 D11S2243E RPP2
P04843	9.78E−05	0.0235	0.16	1.11	RPN1
V9GYM3	1.74E−04	0.0317	0.70	**1.63**	**APOA2**
Q04760	1.76E−04	0.0317	−0.23	1.18	GLO1
O43324	2.01E−04	0.0322	−0.55	1.46	EEF1E1 AIMP3 P18
P61970	3.99E−04	0.0576	−0.33	1.25	NUTF2 NTF2
A0A087WT59	4.97E−04	0.0632	0.75	**1.68**	**TTR**
P05091	5.54E−04	0.0632	−0.24	1.18	ALDH2 ALDM
Q9BUF5	5.83E−04	0.0632	1.46	**2.75**	**TUBB6**
Q5VV42	6.12E−04	0.0632	0.95	**1.93**	**CDKAL1**
Q99832	7.06E−04	0.0680	−0.14	1.10	CCT7 CCTH NIP7-1
P13647	0.001	0.0896	−0.76	*1.69*	*KRT5*
P31949	0.0011	0.0896	−0.24	1.18	S100A11 MLN70 S100C

Bold: > 1.6fold upregulated; *italic* > 1.6fold downregulated.

phase CD sera. GO analysis (GOterm_BP_all) of 17 regulated proteins resulted in GO-terms given in the supplementary data Table d (p value < 0.06). Mainly TTR, APOA1 and APOA2 were classified into the most significant GO terms. The KEGG_pathway analysis provided by DAVID analysis software identified 2 proteins each to belong to the terms "pyruvate metabolism", "pathogenic E. coli infection" and "PPAR signaling pathway" (APOA1 and APOA2). The reactome pathway analysis identified 8 pathways (R-HSA-389957, R-HSA-975634, R-HSA-1799339, R-HSA-390450, R-HSA-174800, R-HSA-389960, R-HSA-5626467, R-HSA-437239). The pathways mainly included the proteins ACTB, TBB6 and CCT7, all involved in protein folding and cytoskeleton forming.

This analysis shows that in this early differentiation stage of the cells, CD sera after 14 days predominantly influenced proteins of basic cell metabolism.

Comparative analysis between healthy control serum in the presence or absence of cytokines. Due to the actual believe, that IL-1, IL-6 and TNF alpha are the most important cytokines in CD, we used a fixed combination as a control. The concentration we adapted to the cytokine levels from our CD patients. If Proteins identified by the cytokine combination would be the same as with CD serum in SP, the cytokine combination would be the cause.

We identified 17 proteins which were differentially expressed in SCP-1 cells after application of healthy control serum supplemented with cytokines compared to healthy control without added cytokines (cutoff Padjust < 0.05-fold changes < 1.6). This result must be valuated as explorative because only three technical replicates of one biological sample were compared to three technical replicates of another biological sample.

Four proteins appeared to be significantly (p < 0.05) more than 1.6-fold upregulated caused by the addition of cytokines: SOD2 (NO-Synthesis), CAMK2D (Ca-signaling), TARDBP (regulates transcription and splicing), RPL7 (ribosomal protein). Thirteen proteins were significantly downregulated KRT1, KRT5, KRT6A, KRT10 (all keratins), SLC44A2 (sugar/choline transport), PTGIS (cytochrome 450 protein), ARL3 (GTP-binding protein), H2AFX (replication independent histone), TSPO (steroid hormone synthesis), ACO1 (aconitase; iron metabolism), PSMB10 (proteasome), PLEKHO2 (innate immune system) and RPS13 (ribosomal protein) (Table 4).

Although we could not find a significant difference in cytokine concentrations (IL-6, TNF alpha or IL-1 beta) in our sera samples obtain during symptomatic or remission phase, we compared the protein expression identified by the comparative analyses (symptomatic phase vs remission (a) to the healthy control sera (pure and plus addition of cytokines) and vice versa.

As a result we found comparable changes in protein expression when we look at the protein expression of TTR, TUBB6 and CDKAL1, but not for APOA1 and APOA2 or KRT10 in cells treated with or without cytokines (Fig. 3). In addition, proteins that were regulated by addition of cytokines to healthy control serum (Table 4) were not affected comparing symptomatic phase versus remission sera. This comparison was depicted to illustrate that the change in expression of these proteins can be caused by disease phases and to some extent by the effect of increased cytokines (Fig. 3 and 4).

These comparisons show, that only in part the three cytokines cause the changes in SCP-1 cells (e.g.KRT6A) and that therefore other factors in the serum of CD patients may be also involved, indeed.

GO analysis of proteins, regulated by the addition of cytokines to a healthy non CD serum. GO analysis (GOterm_BP_all) of 17 regulated proteins resulted in the GO-terms (p value < 0.01) given in supplementary data Table e. Several GO-terms are related to nitric oxide processes. The
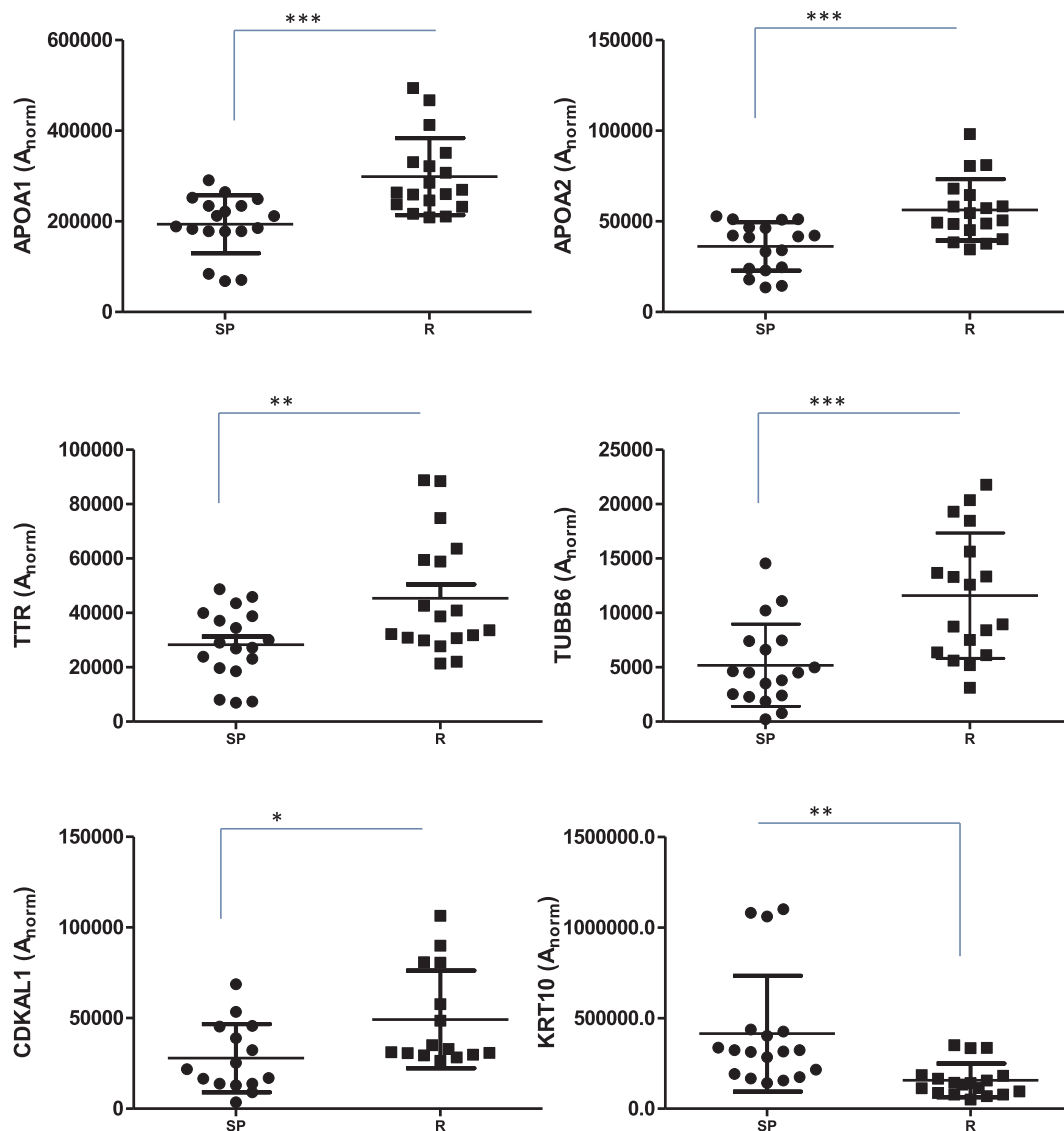
Fig. 1. Protein expression (normalized area) in SCP-1 cells after incubation with CD sera: Sera classified as CD symptomatic phase or remission sera (N = 6; n = 3) N = experimental replicates. n = technical replicates; $^{*} \sim p < 0.05$. $^{**} \sim p < 0.01$ (unpaired t-test) Scattered dot plot with mean and SD.

KEGG_pathway analysis provided by DAVID analysis software identified no single pathway. The reactome pathway analysis gave 6 pathways (R-HSA-156902; R-HSA-192823; R-HSA-2408557; R-HSA-72764; R-HSA-975956; R-HSA-72689). All pathways are involved in ribosomal mechanisms and protein translation. Again, we can show that 14 days in early differentiation of SCP-1 cells mainly induce cell cycle effects.

Comparative analysis between healthy control serum (plus additive cytokines) and CD-sera. The comparison should now reveal those proteins, which are induced by other factors in CD serum independent from the combination of the three cytokines.

We identified 63 proteins, which were significantly ($p < 0.05$) differentially expressed in SCP-1 cells after application of healthy control serum supplemented with cytokines (3 technical replicates) compared to the CD sera (N = 12; n = 3) (cutoff Padjust < 0.05-fold changes < 1.6). Six proteins appeared to be significantly more than 1.6-fold upregulated by the CD sera: GLUD1, CAMK2D (both Fig. 4), RPL18, CAVIN1, RPL13, RPL10 and 19 significantly more than 1.6-fold downregulated: SLC44A2 (Fig. 4), MAPK14, IFITM3, GBP1, EIF4A1, ARMT1, SSR4, BOLA2, TIGAR, PSMC1, GOLT1B, NPC1, TSPO, MAVS, LRRC47, GPX8, CTSL, HUWE1, MPST. These data on the protein

expression of e.g. GLUD1, CAMK2D and SLC44A2 show now in direct comparison clearly, that besides the cytokines IL-6, IL-1 beta and TNF alpha there must be other factors involved, and that these unknown factors also influenced SCP-1 cells (Fig. 4).

However, these results must be valuated as explorative because only three technical replicates of one biological sample were compared to 12 biological replicates of the CD sera group. We therefore refrain from further interpretation of these data.

Comparison of osteoporosis-positive samples to osteoporosis negative samples and to healthy controls. For the development of osteoporosis in our patients are two mechanisms possible. If length of the disease was the responsible factor, then proteins in those with and without osteoporosis should show no difference. However, if there are other, e.g. individual serum factors responsible for bone affection, the sera of those with and without decreased BMD might show different effect on SCP-1 cells.

Eleven significantly (Padjust < 0.05) regulated proteins could be identified in cells incubated with osteoporosis positive versus osteoporosis negative sera (Padjust < 0.05; Table 5). One protein, FABP3 (fatty acid binding protein 3), was more than 1.6fold upregulated in SCP-1 cells treated with osteoporosis positive sera. Six proteins, HBB,
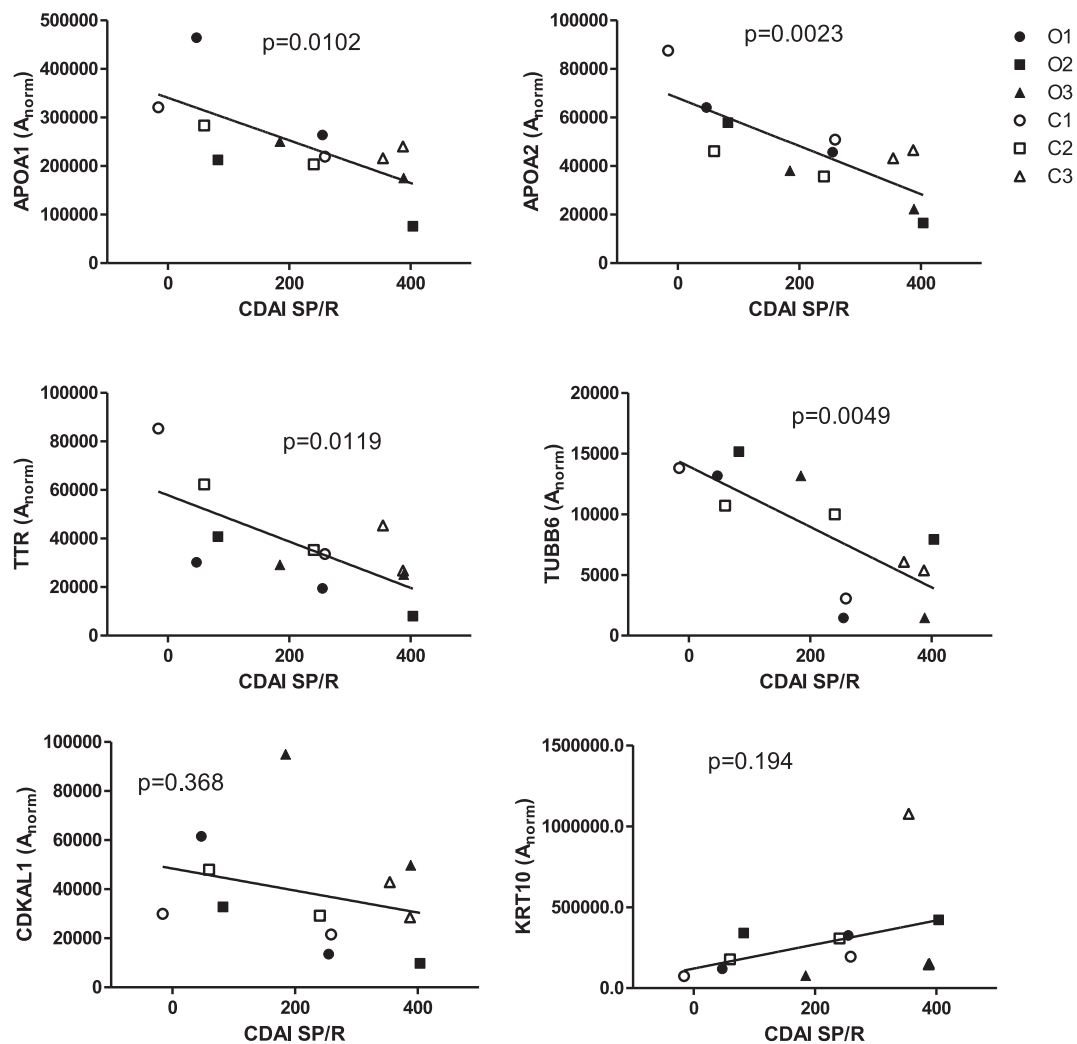
Fig. 2. Mean protein expression (normalized area; n = 3) in SCP-1 cells after incubation with CD sera (osteoporosis positive sera (patient O1, O2, O3) are depicted as black symbols; osteoporosis negative sera (C1, C2, C3) displayed as open symbols): Correlation of CDAI as determined during disease phases (symptomatic and remission phase) to mean protein expression of three technical replicates of protein expression (n = 3).

HBA1/2, JCHAIN, TTR, A2M and FTH1 (hemoglobin beta, hemoglobin alpha, immunoglobulin J, transthyretin, alpha-2 macroglobulin and ferritin heavy chain) were more than 1.6fold downregulated.

A graphical presentation of the changes in normalized protein expression of FABP3, HBB, HBA1/2, TTR, A2M, Jchain and FTH1 in SCP-1 cells treated with osteoporosis positive sera versus osteoporosis negative sera is given in Fig. 4. A graphical presentation of the comparison of protein expression after application of CD patient sera to healthy control sera is also given in Fig. 5. The normalized protein expression of FABP3 (n = 18) was significantly upregulated in SCP-1 cells by the osteoporosis positive sera (2.2fold) and also upregulated compared to the healthy control sera (n = 6; 2.2fold). No difference in protein expression could be seen comparing the osteoporosis negative sera (n = 18) to the healthy control sera (n = 6). The proteins HBB and HBA1/2 were significantly downregulated by the osteoporosis positive sera (HBB 2.2fold; HBA1/2 2.6fold) and also downregulated compared to the healthy control sera (HBB 2.7fold; HBA1/2 3.3fold) (Fig. 5). The normalized protein expression of TTR, FTH1, JCHAIN and A2M was significantly downregulated by osteoporosis positive sera (1.7fold, 3.2fold, 2.5fold, 2.3fold, respectively) while no significant changes could be determined in comparison to the healthy control sera.

These data show that there is a relevant difference between the sera of those with osteoporosis and of those with normal BMD.

To further verify our data, we investigated a possible linear relationship of BMD and protein expression of the identified proteins in SCP-1 cells. Hence, we plotted the BMD femoral neck values in g/cm^2 (BMD spine is given in supplementary data Fig. D) versus the normalized mean protein expression (Fig. 6).

For FABP3 and TTR a significant linear relationship could be determined (p = 0.0026, r^2 = 0.61; p = 0.035, r^2 = 0.37, respectively), indicating a linear relationship of these proteins to increasing BMD. For HBB and HBA1 the linear relationship of BMD to protein expression was not significantly different from zero (HBB: r^2 = 0.19; HBA1: r^2 = 0.24).

The graphical presentation of mean protein expression for A2M, FTH2 and Jchain protein expression revealed outliers based on the protein expression of some patient sera and no correlation of these proteins to BMD was detectable.

David functional annotation analysis of proteins, regulated by osteoporosis positive versus osteoporosis negative CD sera. The 11 proteins showing increases or decreases (osteoporosis positive versus osteoporosis negative) were subjected to the DAVID functional annotation tool [31].

GO analysis (GOterm_BP_all) of eleven regulated proteins resulted in the most significant GO-terms (p value < 0.001): receptor mediated endocytosis, transport and single organism transport (complete Table of identified GO-terms: supplementary data Table f) The KEGG_pathway analysis provided by DAVID analysis software identified 2 proteins (HSD17B12 and FASN) to belong to the term "fatty acid metabolism".

Table 4
Proteins identified comparing SCP-1 cells treated with healthy control sera versus healthy control sera supplemented with cytokines IL-6, IL-1 beta and TNF alpha (H + cyt/H; $p < 0.05$).

ProtID	P	Padjust	log2FC	Fold change Up or down	GENES
P04264	1.09E − 06	1.58E − 03	− 1.74	*3.35*	*KRT1 KRTA*
P13645	3.06E − 06	2.21E − 03	− 1.81	*3.52*	*KRT10 KPP* (Fig. 3)
P13647	1.94E − 05	9.36E − 03	− 2.07	*4.19*	*KRT5*
Q8IWA5	4.31E − 05	1.56E − 02	− 2.06	*4.18*	*SLC44A2 CTL2 PSEC0210* (Fig. 4)
Q16647	8.29E − 05	2.00E − 02	− 1.16	*2.24*	*PTGIS CYP8 CYP8A1*
P36405	8.32E − 05	2.00E − 02	− 0.97	*1.97*	*ARL3 ARFL3*
P16104	1.35E − 04	2.33E − 02	− 1.35	*2.55*	*H2AFX H2AX* (Fig. 4)
P04179	1.41E − 04	2.33E − 02	0.86	**1.82**	**SOD2** (Fig. 4)
B1AH87	1.45E − 04	2.33E − 02	− 2.18	*4.54*	*TSPO* (Fig. 4)
O15260-3	1.82E − 04	0.02625928	− 1.41	2.66	
Q13642-5	2.71E − 04	3.56E − 02	0.65	1.57	
P21399	3.12E − 04	3.76E − 02	− 0.70	*1.63*	*ACO1 IREB1*
P40306	4.29E − 04	4.77E − 02	− 1.30	*2.47*	*PSMB10 LMP10 MECL1*
Q8TD55	4.73E − 04	4.88E − 02	− 2.40	*5.29*	*PLEKHO2* (Fig. 4) *PLEKHQ1 PP9099*
P62277	5.32E − 04	4.88E − 02	− 1.33	*2.51*	*RPS13*
E9PF82	5.76E − 04	4.88E − 02	1.14	**2.20**	**CAMK2D** (Fig. 4)
K7EN94	6.10E − 04	4.88E − 02	1.31	**2.48**	**TARDBP** (Fig. 4)
P02538	6.19E − 04	4.88E − 02	− 2.73	*6.63*	*KRT6A K6A KRT6D* (Fig. 4)
P18124	6.41E − 04	4.88E − 02	0.77	**1.71**	**RPL7**

Bold: > 1.6fold upregulated; *italic* > 1.6fold downregulated.

(The terms "African trypanosomiasis" and "malaria" were also identified with 2 proteins involved.) The reactome pathway analysis gave 4 pathways, all related to erythrocyte and heme metabolism (R-HSA-2168880, R-HSA-1247673, R-HSA-1237044, R-HSA-432722). As in all GO-analyses, no bone specific protein changes were identified after 14 days incubation with CD sera in less differentiated SCP-1 cells. However, five pathways out of 16 are characterized by endocytosis or transport mechanisms and two more belong to lipid metabolic processes.

Discussion

In this study, we hypothesized that bone metabolism is influenced by factors in the serum of patients with CD, resulting in osteoporosis in some of these patients. Therefore, we analyzed whether serum from CD patients influenced protein expression in a mesenchymal cell line under osteogenic conditions, indicating a change in bone turnover with possible long-term effects. If so, serum from patients with symptomatic CD could possibly have a different effect on SCP-1 cells compared to serum from the same patient in remission. In addition, as an alternative strategy, the effect of serum samples from patients with and without osteoporosis were compared to evaluate differences independent from the acute situation. In addition, we added serum samples from a healthy control donor to the comparative analysis to analyze if the protein expression is affected differentially by CD-sera compared to healthy control sera and if the addition of a cytokine combination IL-6, IL-1 beta and TNF alpha is causing any protein changes.

Analysis of sera from patients with CD showed increased levels of TNF alpha, IL-1 beta and IL-6 during the symptomatic phase compared to healthy patients. These levels differed tremendously between CD
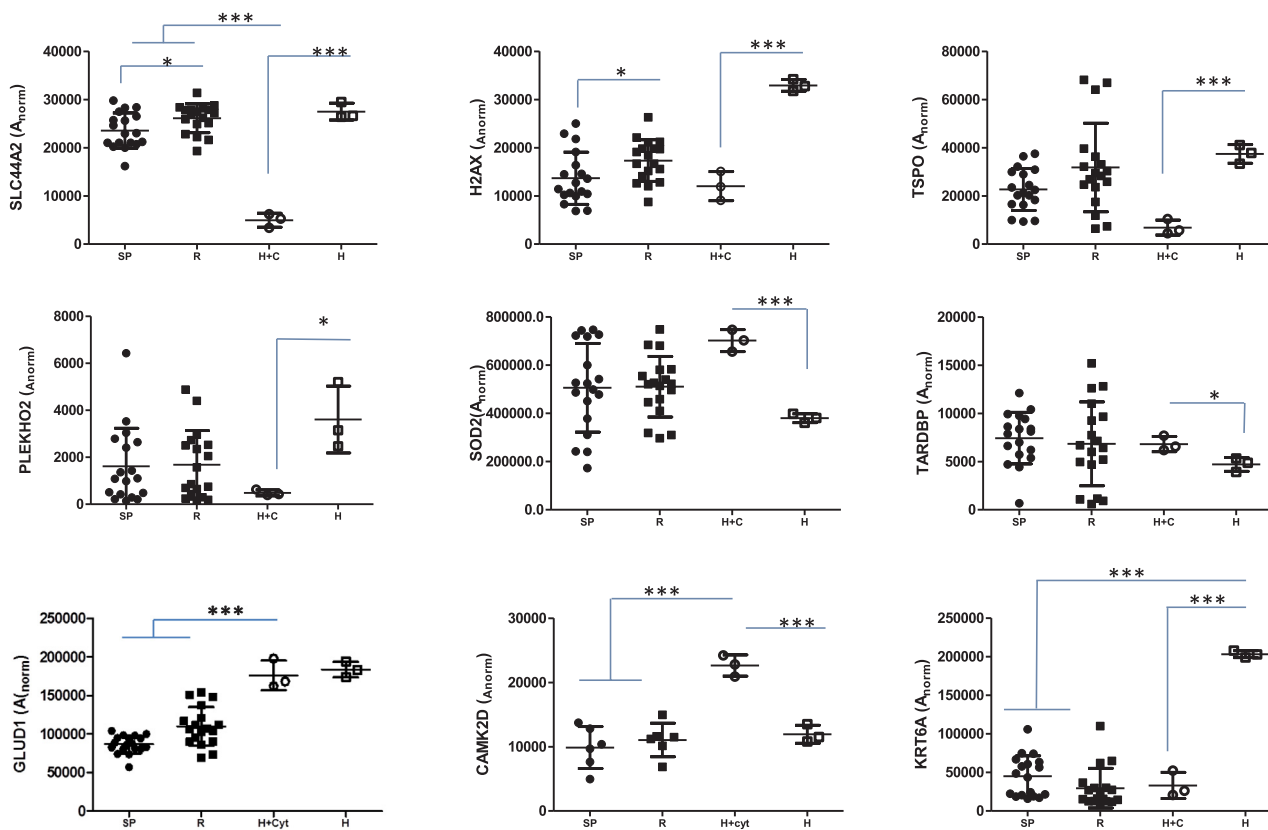

Fig. 3. Protein expression (normalized area) in SCP-1 cells after incubation with CD sera (SP or R) or healthy control sera with (H + C; n = 3) or without addition of cytokines (H; n = 3) CD-Sera were classified as CD symptomatic phase (SP) or remission sera (R) (N = 6; n = 3) N = experimental replicates. n = technical replicates; Scattered dot plot with mean and SD. The first two data sets are identical to Fig. 1 and depicted again for better understanding of the comparative analysis to the healthy controls.
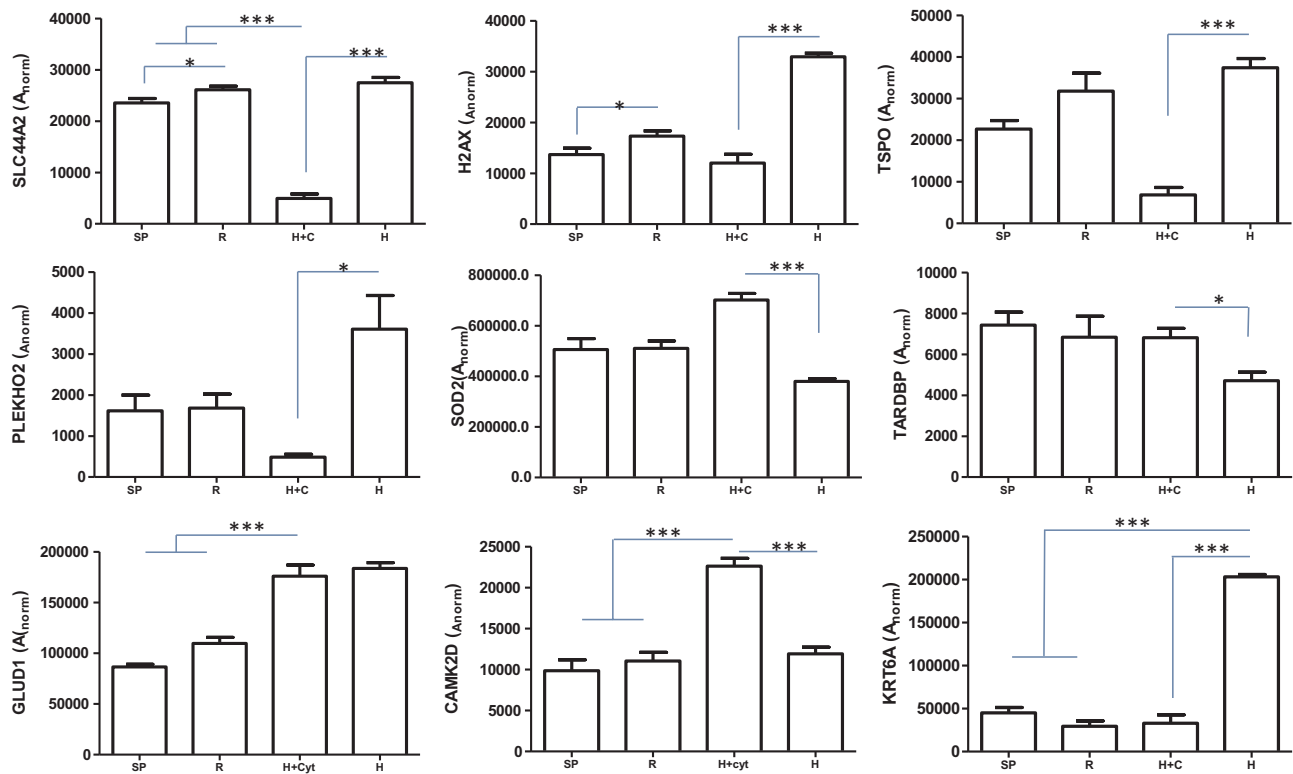
Fig. 4. Protein expression (normalized area) in SCP-1 cells after incubation with CD sera (SP or R) or healthy control sera with (H + C; n = 3) or without addition of cytokines (H; n = 3) CD-Sera were classified as CD symptomatic phase (SP) or remission sera (R) (N = 6; n = 3) N = experimental replicates. n = technical replicates $^*{\sim}\mathrm{p} < 0.05$, $^{**}{\sim}\mathrm{p} < 0.01$, $^{***}{\sim}\mathrm{p} < 0.001$ (unpaired *t*-test or One way Anova (H + cyt vs CD-sera (SP + R)) Scattered dot plot presenting mean and SD.

Table 5
Significantly regulated proteins in SCP-1 cells after treatment with osteoporosis positive CD sera (Ost + /Ost −; p < 0.05).

ProtID	P	Padjust	log2FC	Fold change up or down	GENES
P68871	1.00E − 07	8.69E − 05	−1.18	2.27	*HBB*
P69905	1.20E − 07	8.69E − 05	−1.61	3.06	*HBA1/2*
A0A087X2C0	3.30E − 07	1.58E − 04	−1.68	3.21	
S4R371	7.20E − 07	2.61E − 04	0.71	1.63	**FABP3**
D6RHJ6	4.10E − 06	1.19E − 03	−1.80	3.49	*JCHAIN*
A0A087WT59	8.49E − 06	2.04E − 03	−1.08	2.11	*TTR*
P02786	4.03E − 05	0.0075	0.34	0.79	TFRC
C9JF17	4.14E − 05	0.0075	−0.61	1.53	APOD
P49327	6.95E − 05	0.0108	0.41	0.75	FASN FAS
Q53GQ0	7.50E − 05	0.0108	0.30	0.81	HSD17B12 SDR12C1
P01023	8.62E − 05	0.0113	−0.71	1.63	*A2M* CPAMD5 FWP007
P02794	2.95E − 04	0.0355	−1.57	2.97	*FTH1* FTH FTHL6 OK/SW-cl.84 PIG15

Bold: > 1.6fold upregulated; *italic* > 1.6fold downregulated.

patients, as also reported by Turk et al. [15]. No single cytokine level correlated to BMD in our patients. IL-6, the main contributing cytokine to CD in children, as discussed by Sylvester [17], was not consistently increased in the serum of patients in this study. Furthermore, no gender specific increased IL-6 level was observed. In contrast all female CD patients were found to have increased TNF alpha levels. A gender-specific influence of especially IL-6 on modulation of the adrenal response and the HPA axis was postulated by Bethin et al. They assumed that this could be one reason for a higher prevalence of autoimmune diseases in female [37]. Statistical analysis of serum parameters revealed no significantly changed concentrations of analyzed cytokines

IL-6, IL-1 beta or TNF alpha, neither between active disease and remission nor between serum from osteoporosis positive and negative patients. This was caused by the distinct differences in cytokine levels between the studied individuals. The mean values for IL-6 and TNF alpha were in the same range as published by Ogawa et al. [38]. In our former study analyzing cytokine values in CD patients the comparison of cytokine levels with age and gender matched healthy controls revealed about 10fold elevated cytokine levels for all three cytokine analyzed [13].

We found lower BMD values with longer duration of CD in the studied patients. This finding suggests that the time period bone is exposed to CD sera is involved in the pathogenesis of osteoporosis over the years. Data on GC therapy concerning daily dose, duration or cumulative dose and BMD are contradictory to date [14,39–42]. GC cumulative dose in part correlated to BMD in single disease studies. However in some studies a correlation only for fractures was evident [42]. As shown also in our patients with different diseases the duration or the cumulative glucocorticoid dosage per se were not causal for decreased BMD [43]. BMD is only in part responsible for bone disease in GC therapy [11].

CDAI values were significantly increased during the symptomatic phase compared to remission supporting the successful categorization. Although not all clinicians support the use of this clinical grading in CD, it was applied during the time of this study in Germany. Using proteome analysis to compare the effect of sera defined by CDAI phases on SCP-1 cells was a proof of concept that factors in serum of CD patients influence bone.

This analysis comparing symptomatic phase sera to remission sera revealed seven proteins to be relevant for bone metabolism in SCP-1 cells therefore also for bone turnover in patients with CD. Five proteins (TUBB6, CDKAL1, TTR, APOA1 and APOA2; Table 3; Figs. 1 and 2) were significantly more than 1.6-fold downregulated and two proteins (2 keratins) were significantly upregulated by symptomatic phase sera.
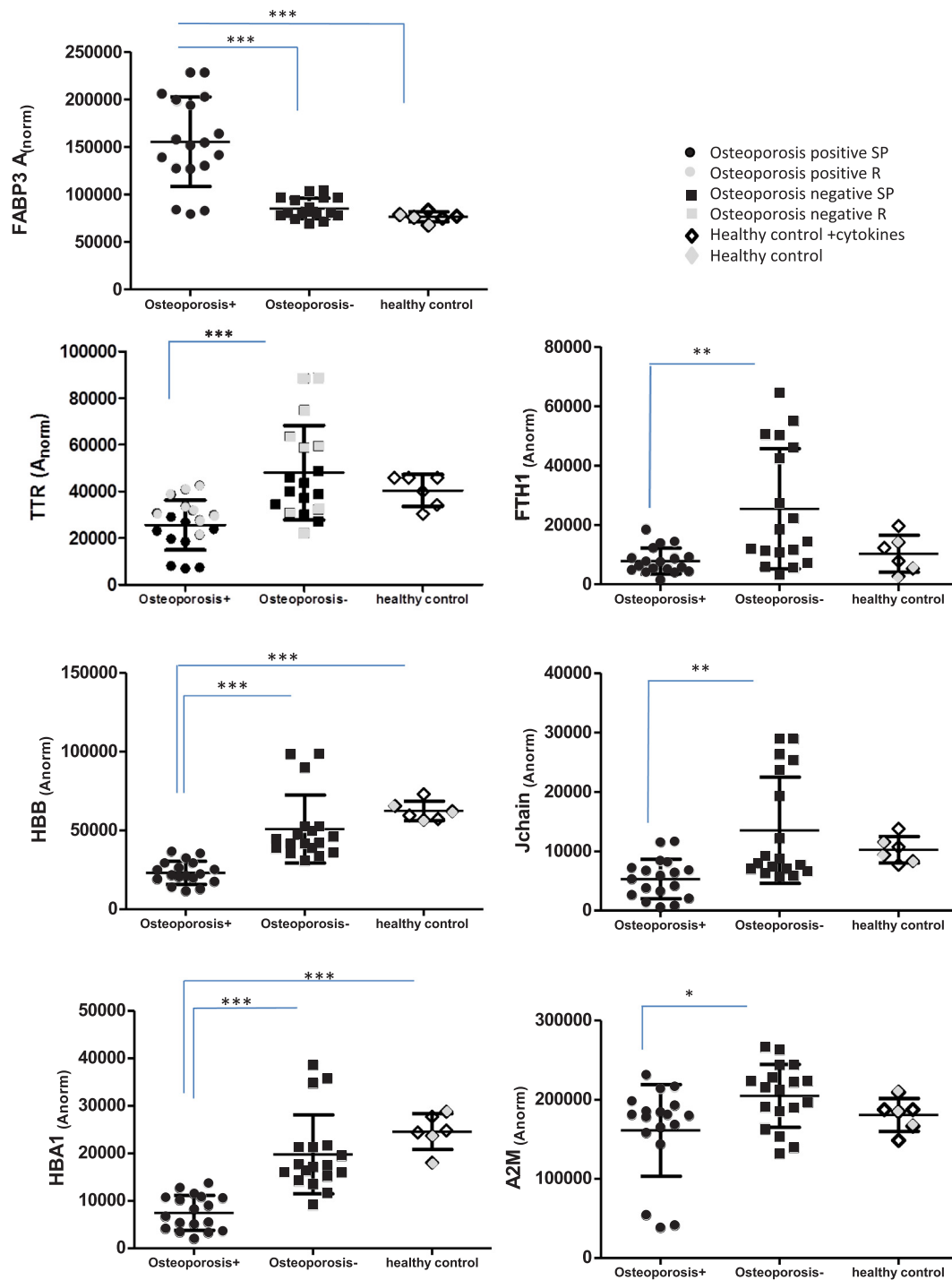
Fig. 5. Protein expression (normalized area) in SCP-1 cells after incubation with CD patient sera or healthy control sera: Osteoporosis positive sera (Symptomatic phase (black) and remission sera (grey); N = 6; n = 18), osteoporosis negative sera (Symptomatic phase (black) and remission sera (grey); N = 6; n = 18) and healthy control sera (with (open) or without added cytokines (closed symbols) N = 2; n = 6). N = experimental replicates. n = technical replicates; $^{*} \sim p < 0.05$, $^{**} \sim p < 0.01$ (One-way Anova followed by Bonferroni multiple comparison testing).

These proteins had not been associated with osteoporosis or bone turnover so far and the basic expression of these proteins was low. However, considering that protein expression increased within the 14 days of incubation, it is probable that longer and even higher exposure of increased protein levels would affect osteoblasts. This hypothesis is supported by the finding, that patients with longer duration of CD showed lower BMD.

Furthermore, a significant linear relation between the CDAI level and the protein expression of TUBB6, TTR, APOA1 and APOA2 was found. This strengthens the finding that these proteins were expressed depending on the degree of CD and vary in their expression between symptomatic and remission phase defined by CDAI.

This change in protein expression could not solely be caused by the elevated levels of the cytokines IL-6, IL-1 beta and TNF alpha during symptomatic phase, because we did not find a significant difference between cytokine levels comparing disease phases. However, there was indeed a difference between stimulation with these three cytokines compared to all CD-sera. Indicating that besides the increased levels of
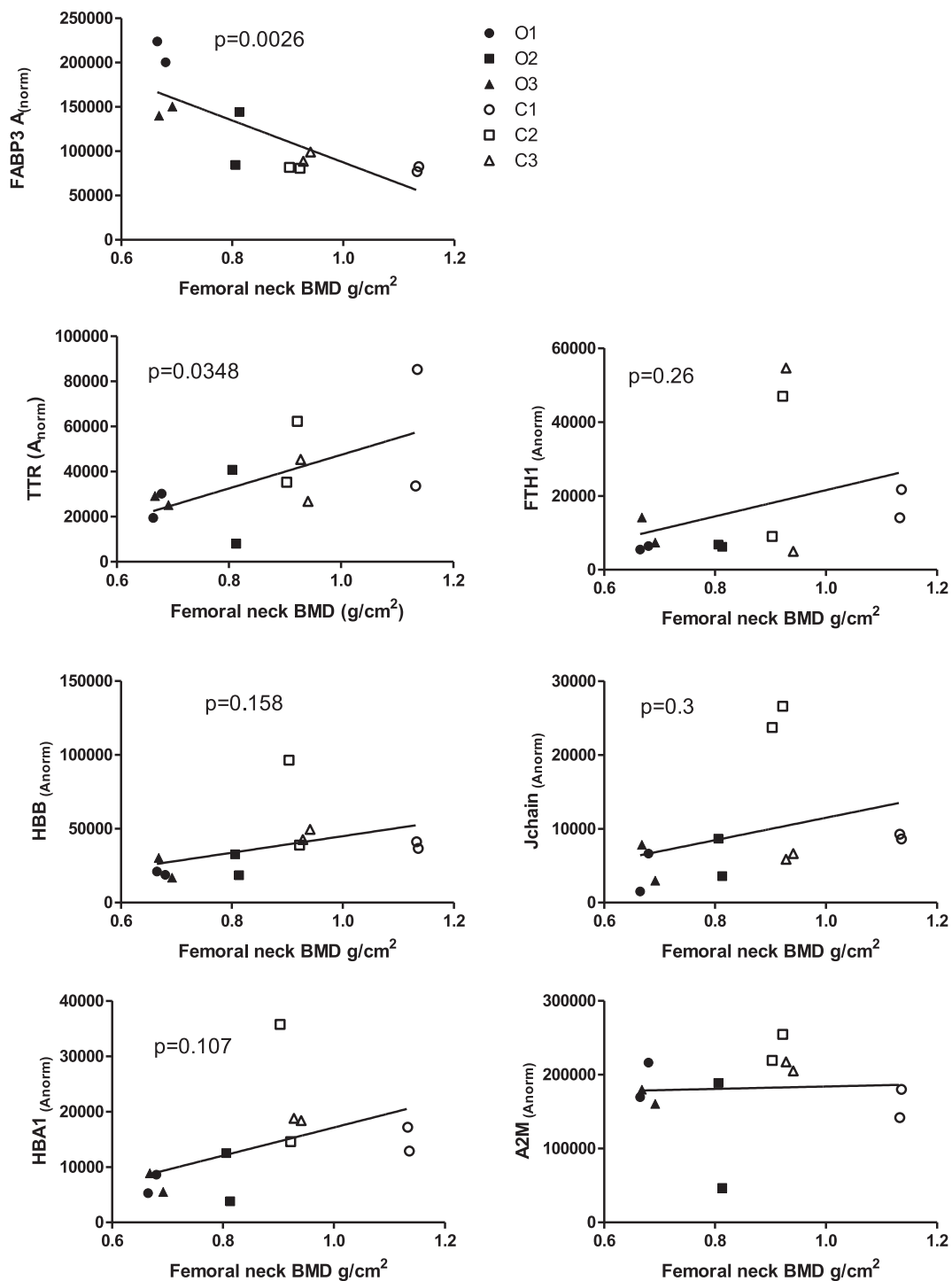
Fig. 6. Mean protein expression (normalized area) in SCP-1 cells after incubation with CD patient sera (osteoporosis positive sera (patient O1, O2, O3) are depicted as black symbols; osteoporosis negative sera (C1, C2, C3) are open symbols). Correlation of BMD at femoral neck (g/cm^2) as measured during symptomatic and remission phase to mean protein expression of three technical replicates of protein expression (n = 3).

cytokines, additional factors in CD sera caused these changes in protein expression (Figs. 3 and 4). The GO-analysis including proteins identified by remission versus symptomatic phase sera analysis revealed only 3 or 2 proteins (TTR, APOA1 and APO2) in every identified GO-term. Some of these identified pathways can be connected to inflammation. No data of these proteins in correlation to inflammation in CD were published to date. However, these proteins were identified also in cholesterol and lipoprotein metabolisms pathways which affect cell membrane composition and therefore influence the uptake of serum factors, especially inflammatory mediators [44,45]. Therefore,

principally an effect of CD serum on inflammatory pathways can be shown.

The most surprising was the distinct influence of CD serum from patients with and without osteoporosis on our cell model. The most noticeable proteins induced by osteoporosis positive sera were FABP3, whereas HBB as well as HBA1/2 were decreased, all three proteins showing a strong relationship to BMD levels.

Overexpression of FABP3 inhibited mesenchymal stem cell proliferation and FABP3 increased during hypoxic conditions [46]. Until now neither nor HBB or HBA1/2 had been known to be related to bone

metabolism. These proteins are involved in mechanisms of oxygen transport and hypoxia.

Several studies have shown that hypoxia affected differentiation in human MSCs and can inhibit osteogenesis [47,48]. However, other studies have shown that during early differentiation hypoxic conditions favor osteogenic rather than adipogenic differentiation [49]. In another study using a rat model, hypoxia also induced differentiation towards osteogenesis in the initial stages of differentiation, but long term exposure to hypoxic conditions led to impaired osteogenesis in the later stages of differentiation [50].

In our experimental set-up we did not use defined hypoxic conditions. In addition, besides a basal differentiation with glycerophosphate and vitamin C no medium supplements to induce osteogenic (e.g. vitamin D or dexamethasone) or adipogenic differentiation (e.g. IBMX and dexamethasone) was used. Therefore, in this early differentiation stage no induction of adipogenic or osteogenic phenotype was detectable in SCP-1 cells.

As reported before of the use of osteoporosis positive sera compared to exposure of osteoporosis negative sera in hMSC increased mRNA levels of osteopontin (SPP1), a marker for osteogenesis. In addition, RANKL/OPG ratio was increased suggesting increased osteoclast activation [51]. HMSCs have a raised differentiation potential compared to SCP-1 cells. Our published results in hMSCs would support, that principally the observed changes in protein levels of FABP3, HBB and HBA1/2 in SCP-1 might favor the development of osteoporosis.

GO-analysis performed with the proteins after stimulation with osteoporosis positive sera or osteoporosis negative sera resulted in the GO-pathways: endocytosis, intracellular transport mechanisms as well as lipid metabolisms. Especially the endocytosis pathway was found to play a major role in inflammatory bowel diseases [52,53]. All identified pathways are involved in cytokine mediated changes in tight junction and adherent junction of intestinal cells [54], although none of the identified proteins e.g. FABP3 were in the focus of research so far. Our data suggest that also proteins of early bone cell differentiation may be mediators of bone affection in inflammatory bowel disease.

Taken together, our study describes for the first time the protein expression pattern in a human osteogenic cell system dependent on stimulation with sera from adult patients with CD. Protein levels, more relevant than gene expression for the final phenotype, were clearly linearly affected dependent on CD phase (CDAI). Some proteins also correlated in a linear relationship to patient BMD independent from CD phase, suggesting that additional factors in serum besides IL-6, IL-1 beta and TNF alpha are responsible for the osteoporotic phenotype.

Our findings are limited to patients with CD and should not be transposed to osteoporosis patients without CD. However, in postmenopausal osteoporosis also IL-1, IL-6 and TNF alpha are involved locally in bone turnover, and the frequent and prolonged exposure of bone to inflammatory mediators is one of the main contributors in the formation of this form of estrogen-loss induced osteoporosis. In this study we did not investigate bone from patients with CD. Certainly, turnover or sensitivity of bone in patients with CD may also play an important role for the development of bone affection.

Conclusion

Our results suggest, that serum from patients with CD influenced bone metabolism supporting our hypothesis. Phase of the disease was not the most decisive factor contributing to the osteoporotic phenotype but additional parameters seemed to be involved. The effects on bone metabolism were detectable early in the differentiation period, hence predominantly affecting proteins of the general cell metabolism before the development of an osteoblastic phenotype.

The present study should be regarded as explorative. We intend to give an idea about possible pathways, playing a role in the pathogenesis of osteoporosis in CD. Additional experiments including e.g. si-RNA experiments targeting the hypoxia, endocytosis or lipid metabolism

pathway could be valuable to support our hypothesis.

Ethical considerations

This study was approved by the Ethics Committee of the University Clinic of Goettingen, and informed consent was signed by all subjects.

Author's contributions

Martina Blaschke designed parts of the study, performed parts of the data analysis and prepared the figures and the manuscript. Regine Köpp conducted all experiments. Christof Lenz was responsible for proteome analysis. Jochen Kruppa and Klaus Jung were responsible for statistical data analysis. Heide Siggelkow initiated and designed parts of the study, applied and received grants and was involved in the writing of the manuscript.

Competing interests

The authors have no conflicts of interest to disclose.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcte.2018.06.002.

References

[1] Ghosh S, et al. Low bone mineral density in Crohn's disease, but not in ulcerative colitis, at diagnosis. Gastroenterology 1994;107(4):1031–9.
[2] Mirza F, Canalis E. Management of endocrine disease: secondary osteoporosis: pathophysiology and management. Eur J Endocrinol 2015;173(3):R131–51.
[3] Johnell O, et al. Mortality after osteoporotic fractures. Osteoporos Int 2004;15(1):38–42.
[4] Jalava T, et al. Association between vertebral fracture and increased mortality in osteoporotic patients. J Bone Miner Res 2003;18(7):1254–60.
[5] Schulte CM. Review article: bone disease in inflammatory bowel disease. Aliment Pharmacol Ther 2004;20(Suppl 4):43–9.
[6] Van Staa TP, et al. Use of oral corticosteroids and risk of fractures. J Bone Miner Res 2000;15(6):993–1000.
[7] Vestergaard P, et al. Fracture risk is increased in Crohn's disease, but not in ulcerative colitis. Gut 2000;46(2):176–81.
[8] Vestergaard P, Mosekilde L. Fracture risk in patients with celiac Disease, Crohn's disease, and ulcerative colitis: a nationwide follow-up study of 16,416 patients in Denmark. Am J Epidemiol 2002;156(1):1–10.
[9] Klaus J, et al. High prevalence of osteoporotic vertebral fractures in patients with Crohn's disease. Gut 2002;51(5):654–8.
[10] Lamb EJ, et al. Metabolic bone disease is present at diagnosis in patients with inflammatory bowel disease. Aliment Pharmacol Ther 2002;16(11):1895–902.
[11] Stockbrugger RW, et al. Discordance between the degree of osteopenia and the prevalence of spontaneous vertebral fractures in Crohn's disease. Aliment Pharmacol Ther 2002;16(8):1519–27.
[12] Sakellariou GT, et al. Bone density in young males with recently diagnosed inflammatory bowel disease. Joint Bone Spine 2006;73(6):725–8.
[13] Siggelkow H, et al. Erythrocyte sedimentation rate as an osteoporosis risk factor in patients with active Crohn's disease. Osteology 2009;18:209–16.
[14] Targownik LE, et al. Inflammatory bowel disease has a small effect on bone mineral density and risk for osteoporosis. Clin Gastroenterol Hepatol 2013;11(3):278–85.
[15] Turk N, et al. Proinflammatory cytokines and receptor activator of nuclear factor kappaB-ligand/osteoprotegerin associated with bone deterioration in patients with Crohn's disease. Eur J Gastroenterol Hepatol 2009;21(2):159–66.
[16] Reinecker HC, et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993;94(1):174–81.
[17] Sylvester FA, et al. Effect of Crohn's disease on bone metabolism in vitro: a role for interleukin-6. J Bone Miner Res 2002;17(4):695–702.
[18] Hyams JS, et al. Alterations in bone metabolism in children with inflammatory

bowel disease: an in vitro study. J Pediatr Gastroenterol Nutr 1997;24(3):289–95.

[19] Stevens C, et al. Tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 expression in inflammatory bowel disease. Dig Dis Sci 1992;37(6):818–26.

[20] Blaschke M, et al. IL-6, IL-1beta, and TNF-alpha only in combination influence the osteoporotic phenotype in Crohn's patients via bone formation and bone resorption. Adv Clin Exp Med 2018;27(1):45–56.

[21] Best WR, et al. Development of a Crohn's disease activity index. National cooperative crohn's disease study. Gastroenterology 1976;70(3):439–44.

[22] Vihinen MK, et al. Bone turnover and metabolism in paediatric patients with inflammatory bowel disease treated with systemic glucocorticoids. Eur J Endocrinol 2008;159(6):693–8.

[23] Schieker M, et al. Human mesenchymal stem cells at the single-cell level: simultaneous seven-colour immunofluorescence. J Anat 2007;210(5):592–9.

[24] Blaschke M, et al. IL-6, IL-1β, and TNFα-only in combination- influence the osteoporotic phenotype in Crohn's patients via bone formation and bone resorption. J Adv Clin Exp Med 2016. manuscript accepted.

[25] Yu YQ, et al. Enzyme-friendly, mass spectrometry-compatible surfactant for in-solution enzymatic digestion of proteins. Anal Chem 2003;75(21):6023–8.

[26] Lambert W, et al. Probing the transient interaction between the small heat-shock protein Hsp21 and a model substrate protein using crosslinking mass spectrometry. Cell Stress Chaperones 2013;18(1):75–85.

[27] Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3. p. Article3.

[28] Benjamini Y, et al. Controlling the false discovery rate in behavior genetics research. Behav Brain Res 2001;125(1–2):279–84.

[29] Jung K, Friede T, Beissbarth T. Reporting FDR analogous confidence intervals for the log fold change of differentially expressed genes. BMC Bioinf 2011;12:288.

[30] Beissbarth T, Speed TP. GOstat: find statistically overrepresented Gene Ontologies within a group of genes. Bioinformatics 2004;20(9):1464–5.

[31] Huang DW, et al. DAVID bioinformatics resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res 2007;35:W169–75. Web Server issue.

[32] Sostegni R, et al. Review article: Crohn's disease: monitoring disease activity. Aliment Pharmacol Ther 2003;17(Suppl 2):11–7.

[33] Bocker W, et al. Introducing a single-cell-derived human mesenchymal stem cell line expressing hTERT after lentiviral gene transfer. J Cell Mol Med 2008;12(4):1347–59.

[34] Owen TA, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J Cell Physiol 1990;143:420–30.

[35] Siggelkow H, et al. Development of the osteoblast phenotype in primary human osteoblasts in culture: comparison with rat calvarial cells in osteoblast differentiation. J Cell Biochem 1999;75(1):22–35.

[36] Siggelkow H, et al. Evidence of downregulation of matrix extracellular phosphoglycoprotein during terminal differentiation in human osteoblasts. Bone

2004;35(2):570–6.

[37] Bethin KE, Vogt SK, Muglia LJ. Interleukin-6 is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation. Proc Natl Acad Sci USA 2000;97(16):9317–22.

[38] Ogawa K, et al. Profiles of circulating cytokines in patients with Crohn's disease under maintenance therapy with infliximab. J Crohns Colitis 2012;6(5):529–35.

[39] Ardizzone S, et al. Altered bone metabolism in inflammatory bowel disease: there is a difference between Crohn's disease and ulcerative colitis. J Int Med 2000;247(1):63–70.

[40] Braz NFT, et al. Negative impact of high cumulative glucocorticoid dose on bone metabolism of patients with myasthenia gravis. Neurol Sci 2017;38(8):1405–13.

[41] Ceccato F, et al. Long-term glucocorticoid effect on bone mineral density in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Eur J Endocrinol 2016;175(2):101–6.

[42] Walsh LJ, et al. The impact of oral corticosteroid use on bone mineral density and vertebral fracture. Am J Respir Crit Care Med 2002;166(5):691–5.

[43] Siggelkow HBM, Armbrecht G, Thomasius F, Bock O, Tzvetkov M, Gluer CC, Felsenberg D. Genetic variations in the HSD11B1 gene show associations with bone mineral density. In: Osteol Meeting-Schattauer; 2018. p. P15.

[44] Catapano AL, Pirillo A, Norata GD. Vascular inflammation and low-density lipoproteins: is cholesterol the link? A lesson from the clinical trials. Br J Pharmacol 2017;174(22):3973–85.

[45] Graham LS, et al. Bone density and hyperlipidemia: the T-lymphocyte connection. J Bone Miner Res 2010;25(11):2460–9.

[46] Wang S, et al. Overexpression of FABP3 inhibits human bone marrow derived mesenchymal stem cell proliferation but enhances their survival in hypoxia. Exp Cell Res 2014;323(1):56–65.

[47] Yang DC, et al. Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. PLoS One 2011;6(9):e23965.

[48] Fehrer C, et al. Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. Aging Cell 2007;6(6):745–57.

[49] Hung SP, et al. Hypoxia promotes proliferation and osteogenic differentiation potentials of human mesenchymal stem cells. J Orthop Res 2012;30(2):260–6.

[50] Ding H, et al. Continuous hypoxia regulates the osteogenic potential of mesenchymal stem cells in a time-dependent manner. Mol Med Rep 2014;10(4):2184–90.

[51] Blaschke M, et al. Differential expression of osteoblast related genes in mesenchymal progenitor cells induced by serum of patients with active crohn's disease with and without osteoporosis. J Stem Cells Regener Med 2007;2(1):47–8.

[52] Utech M, Mennigen R, Bruewer M. Endocytosis and recycling of tight junction proteins in inflammation. J Biomed Biotechnol 2010;2010:484–987.

[53] Naim HY, Zimmer KP, Nichols B. Molecular biology of the gut. Mol Cell Pediatr 2016;3(1):32.

[54] Yu D, Turner JR. Stimulus-induced reorganization of tight junction structure: the role of membrane traffic. Biochim Biophys Acta 2008;1778(3):709–16.