Original Article

Determination of serum biomarkers in osteoarthritis patients: a previous interventional imaging study revisited

Timothy McAlindon[1], Eckart Bartnik[2], Janina S. Ried[2], Lenore Teichert[2], Matthias Herrmann[2], Klaus Flechsenhar[2, ✉]

[1] Tufts Medical Center, Chief, Division of Rheumatology, Boston, MA 02111, USA;
[2] Sanofi-Aventis Deutschland GmbH, Frankfurt am Main D-65926, Germany.

Abstract

To evaluate in an interventional trial on knee osteoarthritis (OA) the level and change of two serum biomarkers and their correlation with imaging parameters. The previously reported interventional OA study (ClinicalTrials.gov: NCT00536302) identified a positive effect of collagen hydrolysate (CH) on cartilage morphology in patients with knee OA using delayed gadolinium enhanced magnetic resonance imaging (dGEMRIC). It was the objective in this research project to evaluate in an interventional clinical trial on knee OA the level and change of two serum biomarkers and their correlation with imaging parameters. In blood samples of study participants, we determined the concentration of procollagen type II N-terminal propeptide (PIIANP) and aggrecan chondroitin sulfate 846 epitope (CS846) at baseline (BL) and at the follow-up (FU) visits at 24 and 48 weeks. We measured the level and change of biomarker concentrations in both study groups, and the correlation of those changes with changes in dGEMRIC. For the biomarker PIIANP, we observed a significantly greater increase in the CH group (29.9% *vs.* 1.2% at week 24, $P = 0.001$). For CS846, the mean concentration was lower among the CH treated participants at 24 weeks (78% *vs.* 96%, $P = 0.045$). Consistent correlations of changes in biomarkers PIIANP and CS846 with changes of the dGEMRIC score could not be observed. In this study, different changes per treatment group, CH and placebo were seen for dGEMRIC and PIIANP BL to 24 weeks FU, but only weak correlations between changes in dGEMRIC and biochemical markers.

Keywords: knee osteoarthritis, interventional trial, magnetic resonance imaging, serum chemical biomarkers

Introduction

We previously reported on an interventional study (NCT00536302)[1] that had been performed in patients with osteoarthritis (OA) of the knee Kellgren Lawrence grade (KLG) I and II. The purpose of that prospective, randomized, placebo-controlled, double-blind 48 week pilot study had been to explore whether collagen hydrolysate (CH), a nutritional supplement which had shown symptom-modifying effects in patients with OA[2-3], would also benefit cartilage morphology. From the pre-clinical perspective, experiments with mice had shown that CH is absorbed through the gastro-intestinal tract, circulates in the blood stream and accumulates in

cartilage tissue[4]. *In vitro* experiments had demonstrated that bovine chondrocytes incubated with CH are stimulated to dose-dependently synthesize type-II collagen and proteoglycans[5]. As the density of proteoglycans can be visualized with the technique of delayed gadolinium enhanced magnetic resonance imaging (dGEMRIC), changes of the dGEMRIC T1 relaxation time in the regions of interest at 24 weeks had been defined as primary endpoint in our study for which 30 participants had been recruited and randomized to CH versus placebo in a 1 : 1 ratio. The changes of the dGEMRIC T1 relaxation time or dGEMRIC score in the regions of interest at 24 weeks had been statistically significant in favor of CH when the treatment groups were compared. According to the study protocol, blood was drawn from the study participants at baseline (BL) and during every follow-up (FU) visit. In this publication, we report on the findings of biomarker measurements at BL, at 24 and at 48 weeks, i.e. the time points when imaging was performed. This will enable us to correlate imaging with biomarker data in the interventional OA trial.

We measured two chemical biomarkers in the blood of study participants, PIIANP and CS846[6]. We selected these two because they are most reflective of the likely effects of CH on cartilage collagen and proteoglycan metabolism. PIIANP is a procollagen propeptide which is cleaved off from the collagen type II precursor molecule. The concentration of PIIANP that is measured in serum reflects type II collagen synthesis and can thus be regarded as an anabolic marker of cartilage matrix synthesis[7]. CS846 (aggrecan chondroitin sulfate 846 epitope) is generated when the collagen and proteoglycan matrix is disrupted in arthritic cartilage. With the increased turnover of aggrecan in OA, the CS846 epitope is released into the blood stream. Thus, CS846 must be considered as a catabolic marker for cartilage matrix[8].

This study maps into the larger aim of identifying biochemical and imaging biomarkers for OA. For example, one of the objectives of the epidemiological study that is presently carried out by the Osteoarthritis Initiative[9] is to correlate imaging with chemical biomarker data in patients with OA. The results of that study, comprising 4,796 subjects, in that regard, i.e. a correlation between imaging and biochemical data, are still pending.

Thus, the objective of this study is to analyze the change of concentration of PIIANP and CS846 measured in serum in a clinical intervention trial with CH in OA patients and how those changes relate to the observed changes in dGEMRIC.

Patients and methods

Study sampling

Prior to its initiation, the study had been submitted to the Tufts Medical Center Institutional Review Board which – upon review of the study protocol– had approved the interventional trial. All study participants had consented to the intervention, i.e. the ingestion of the nutritional supplement CH or placebo, the imaging procedures, collection of clinical data and also the analysis of chemical biomarkers in serum. The study had also been submitted to the Food and Drug Administration (FDA) and had been approved by the agency on the condition that the research project be conducted under an investigational new drug (i.e. IND # 74249). The study had been conducted according to the principles of the Helsinki declaration. The study included 30 participants with mild to moderate knee OA, of which 15 participants were treated with CH and 15 with a placebo. All participants were investigated at BL and two FU visits after 24 and 48 weeks. At each visit, dGEMRIC was measured and blood was drawn. For further details of study sampling and dGEMRIC measurement, we refer to our previous publication[1].

Measurements of biomarkers

Blood was centrifuged, aliquoted and then stored at a temperature of -80°. Biomarkers were measured in the following way: After thawing, samples were centrifuged for 10 minutes at 2,000 g at 4°C and then diluted 1:5 for CS846 and 1:8 for determinations of PIIANP. Measurements of CS864 were performed with the CS846 ELISA kit from IBEX Pharmaceuticals Inc., Québec, Canada (assay sensitivity: 15,6 ng/mL to 1,000 ng/mL). PIIANP was determined with the human PIIANP ELISA kit from EMD Millipore, St. Charles, Missouri, U.S.A. (assay sensitivity: 32,8 ng/mL to 2,100 ng/mL). The PIIANP assay did not show cross reactivity with collagen hydrolysates dissolved in water or in PBS. Samples were measured in triplicate.

Statistical analysis

The primary analysis question was if the mean percentage changes in concentration of PIIANP and CS846 from BL to the FU at week 24 and week 48 were different between the group treated with CH and the placebo group. As second question, differences in means of biomarker concentrations at different time points were analyzed. All pairwise comparisons of change in concentration and concentrations between groups were performed with Wilcoxon-tests, to account

for possible deviation from the normality assumption. For the analysis of change, a multiple test correction for four tests (two biomarkers, two time spans) had to be used and for the pairwise comparison of concentration means a multiple test correction of six (two biomarkers, three time points). Bonferroni correction was applied for both tests, leading to a corrected significance level $0.05/4 = 0.0125$ and $0.05/6 = 0.0083$, respectively.

The observed trends were compared with the change in dGEMRIC, which was reported in the previous publication[1]. Moreover, correlation between change in dGEMRIC and change in biomarker concentration was analyzed by Spearman correlations. All analyses were performed in R[8]. In the following, the mean at a specific time point (BL, 24/ 48 week FU) is noted as $mean_{BL}$, $mean_{24}$, $mean_{48}$ and the mean of percentage change from BL to 24 or 48 weeks FU as $mean_{BL \to 24}$ and $mean_{BL \to 48}$, respectively. The group (placebo, CH) is indicated by superscript, e.g. $mean_{BL}^{placebo}$, $mean_{BL \to 24}^{CH}$.

Results

PIIANP

For PIIANP, the trend in the placebo group was different compared to the treated group (***Fig. 1***, ***Table 1***). The samples in the placebo group remained stable from BL to 24 weeks FU ($mean_{BL \to 24}^{placebo}$: 1.17%) and increased towards the 48 weeks FU ($mean_{BL \to 48}^{placebo}$: 18.67%). Meanwhile, the mean concentration of PIIANP in the CH treated samples increased to the 24 weeks FU ($mean_{BL \to 24}^{CH}$: 29.88%) and stayed stable towards the 48 weeks FU ($mean_{BL \to 48}^{CH}$: 25.91%). The difference in percentage change from BL to 24 week FU between placebo and CH treated group was significant with a *P*-value of 0.0014 (corrected significance threshold of 0.0125). The difference in PIIANP concentration of both groups at the 24 weeks FU was nominally significant but the p-value (0.0086) was slightly above the Bonferroni corrected significance threshold (0.0083).
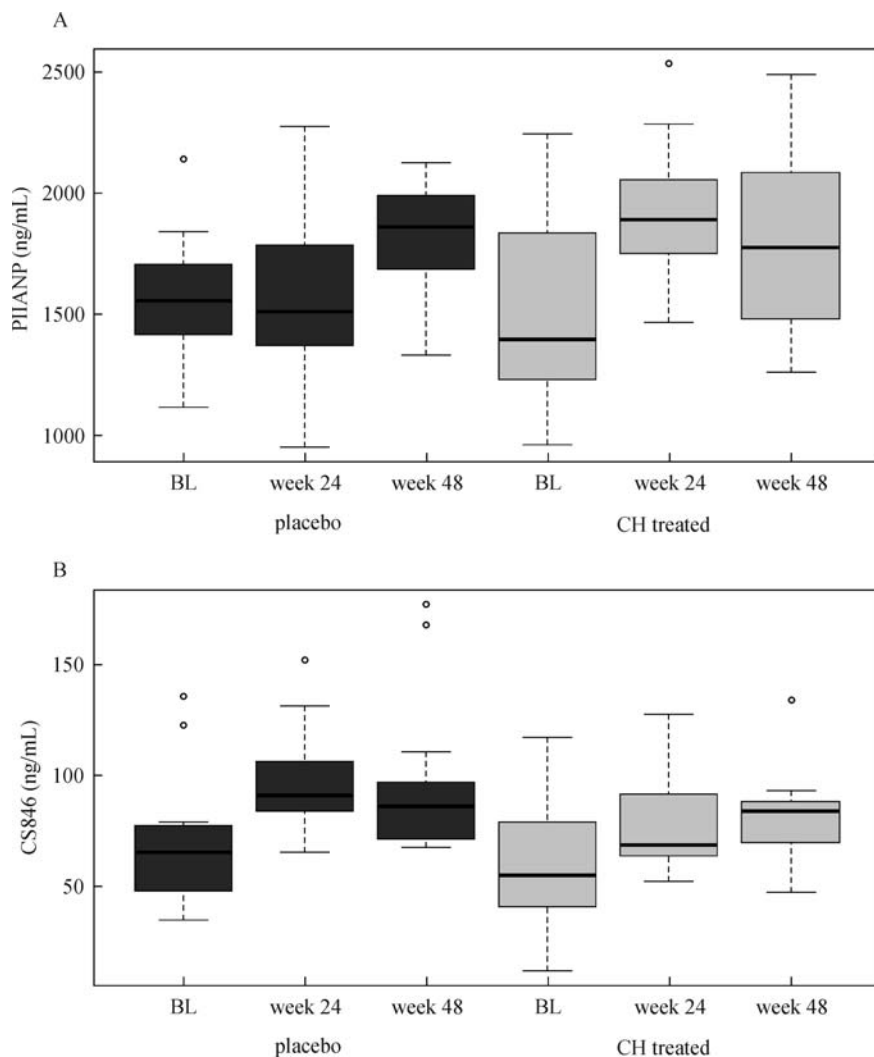

Fig. 1 **Boxplot of PIIANP (A) and CS846 (B) per time point and study arm.**

Table 1   **Levels of PIIANP and CS846 in ng/mL at different time points and change in percentage from base line to 24 weeks follow-up visit (BL → 24) and from base line to 48 weeks follow-up (BL → 48). The *P*-values of the Wilcoxon-test comparing the placebo and CH treated group are given.**

Biomarker		N (BL/24/ 48 weeks)	BL	24 weeks	48 weeks	BL → 24	BL → 48
PIIANP	placebo, mean (SD)	15/15/14	1562.8 (264.62)	1574.17 (337.25)	1816.05 (223.98)	1.17% (16.37)	18.67% (18.17)
	CH treated, mean (SD)	15/15/14	1533.01 (400.44)	1915.16 (288.35)	1829.13 (394.03)	29.88% (26.6)	25.91% (30.74)
	P		0.5393	0.0086	0.8388	0.0014	0.7006
CS846	placebo, mean (SD)	15/15/14	67.53 (29.65)	95.76 (23.07)	95.41 (34.75)	56.79% (48.41)	53.01% (38.19)
	CH treated, mean (SD)	15/15/14	60.17 (29.04)	78.25 (21.04)	81.05 (20.14)	60.55% (102.4)	85.95% (176.34)
	P		0.5949	0.0453	0.2852	0.3453	0.5409

BL: base line, SD: standard deviation, CH: collagen hydrolysate.

The whiskers of the boxplots range to the most extreme points if they are not more than 1.5 times the interquartile range (distance from the first to third quartile = box width) from the box.

CS846:

From the plots in *Fig. 1*, it can be seen that the CS846 concentration developed comparably in both groups. From BL to 24 weeks FU, the CS846 concentration increased and remained relatively stable towards the 48 weeks FU. The mean percentage change from BL to 24 weeks FU ($mean_{BL \to 24}^{placebo}$: 57.79%, $mean_{BL \to 24}^{CH}$: 60.55%) and from BL to 48 weeks ($mean_{BL \to 48}^{placebo}$: 53%, $mean_{BL \to 48}^{CH}$: 86%) were not significantly different between groups. The comparison of mean concentrations between placebo and CH treated participants showed nominally significant higher values for the placebo group for the 24 weeks FU (*P*-value 0.045), which was not significant after correction for multiple testing (significance level 0.0083).

Comparison and correlation with dGEMRIC

In the previous publication on dGEMRIC measurements in the same cohort, a significantly higher increase of dGEMRIC in medial tibia and lateral tibia in the CH treated group was reported for the time span from BL to 24 weeks FU[1]. This difference was not significant for the longer observation period from BL to 48 weeks FU. This trend is comparable to the trend observed for PIIANP. For CS846, no significant differences in change were observed, but nominally significant lower values of CS846 in the CH treated group at time point 24.

The correlation of change from BL to the 24 week FU in PIIANP was positively correlated with the change of dGEMRIC in medial and lateral tibia if placebo and CH treated group are considered combined (Spearman correlation: 0.34/0.36, *Table 2*). Interestingly, the correlation was much weaker and negative if the change from BL to the 48 week FU is considered (-0.13/-0.16). If the placebo and treated group are considered separately, the correlation had the same direction as in the combined analysis apart from the correlation with change in lateral tibia dGEMRIC from BL to 48 week FU. This correlation was negative (-0.41) for the CH treated group and positive for the placebo group (0.21). For CS846, the change was negatively correlated with the change in dGEMRIC for lateral tibia (BL to 24 weeks/48weeks) and medial tibia BL to 48 weeks change and close to zero for medial tibia BL to 24 weeks change (0.02). In contrast to this, the correlations

Table 2   **Spearman correlation between percentage change of biomarker and percentage change of dGEMRIC.**

Biomarker	Treatment group	dGEMRIC medial tibia		dGEMRIC lateral tibia	
		BL → 24	BL → 48	BL → 24	BL → 48
PIIANP	both	0.34	-0.13	0.36	-0.16
PIIANP	placebo	0.06	-0.09	0.31	0.21
PIIANP	CH treated	0.19	-0.09	0.08	-0.41
CS846	both	0.02	-0.16	-0.12	-0.13
CS846	placebo	0.31	-0.01	0.47	-0.23
CS846	CH treated	-0.03	-0.11	-0.26	0.09

BL: base line, SD: standard deviation, CH: collagen hydrolysate.
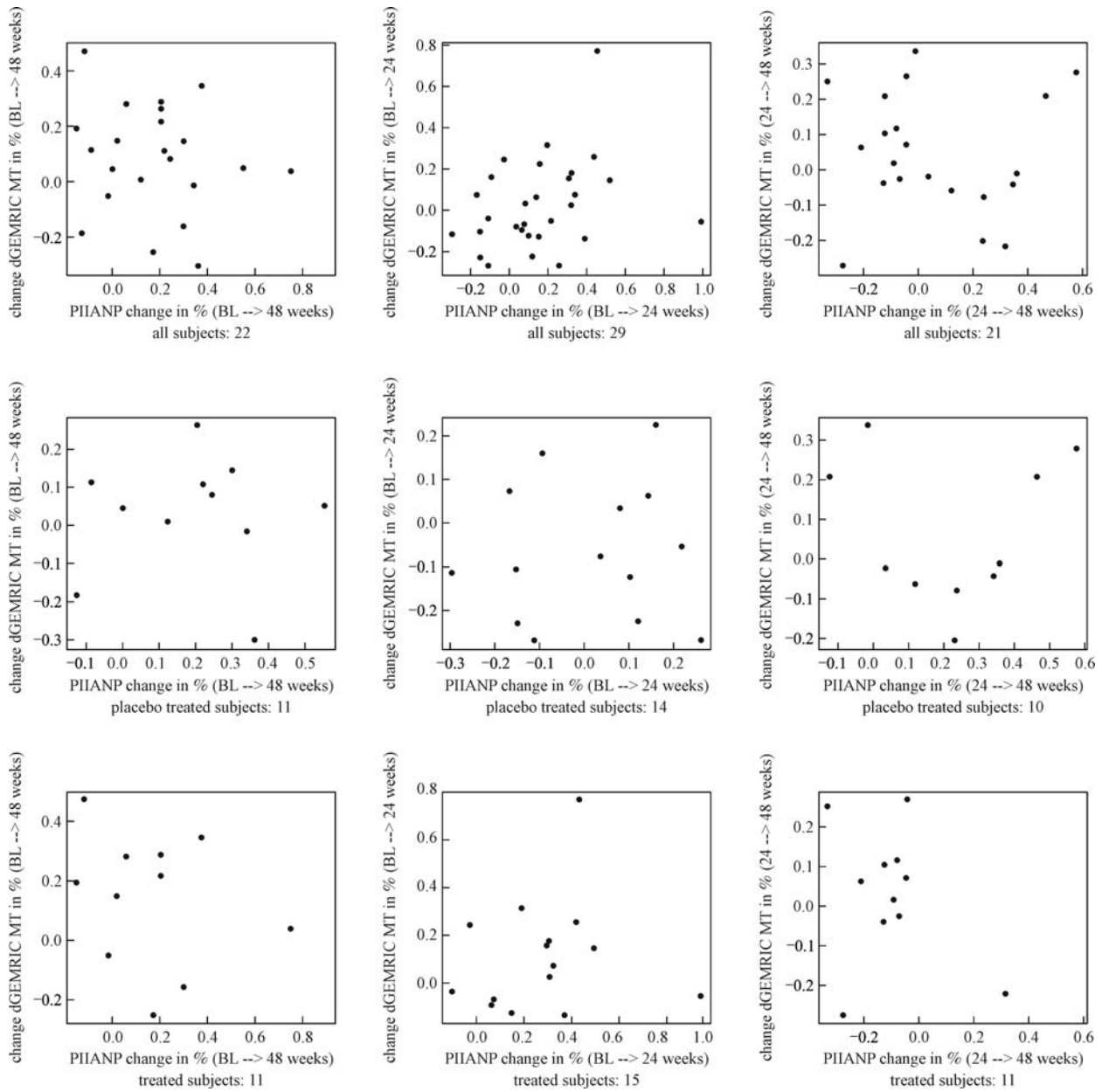
Fig. 2 Correlations between the changes of the dGMERIC scores in the medial tibia and the respective changes of the serum concentration of PIIANP at different time intervals.

of CS846 change in the placebo group from BL to 24 week FU with medial and lateral tibia dGEMRIC change were positively correlated (0.31/0.47).

Correlations are given for the percentage change from base line to 24 weeks follow-up visit (BL → 24) and from base line to 48 weeks follow-up (BL → 48), stratified for placebo ad CH treated group and the combined data set.

Discussion

This study offered a unique opportunity to evaluate two biochemical biomarkers in relation to MRI structural outcome data. In OA research, biochemical markers are evaluated to discriminate subgroups of OA patients in terms of severity of disease, risk of disease progression and response to treatment. So, we are presenting these serum biomarker data in conjunction with a previous positive interventional study. The purpose of this report is to describe to which extent those biomarkers, biochemical and imaging, respond to therapy and how they correlate with each other.

When the changes of the biomarker PIIANP are compared with the changes of the dGEMRIC score, a similar pattern is noted, i.e. increase from BL to 24 weeks and stabilization at 48 weeks in the CH group and stabilization from BL to 24 weeks and increase from 24 to 48 weeks in the placebo group, which means that

changes of both markers, imaging and biochemical, occur in parallel. When the dGEMRIC scores and the PIIANP concentrations are compared between BL and 48 weeks, trends are similar again, which resembles a good fit between imaging and biochemical markers. The fact that both the dGEMRIC score and the PIIANP concentration increase from 24 to 48 weeks in the placebo group may be accounted by the education that study participants were exposed to during the trial. The subjects, assigned to the placebo group, were likely to experience a treatment effect after 48 weeks as they also received recommendations from study personnel how to manage their lives with OA in terms of weight control and physical exercise. When the concentrations of PIIANP and CS846 are compared with the dGEMRIC data of individual patients, then the respective correlations are weak. In general, this weak correlation may be accounted by the fact that in our study we looked at regions of interest in one joint only whereas CH exerts an effect on all the joints which is consequently reflected by the concentration of PIIANP and CS846 in blood. Reference values of serum concentrations of PIIANP and CS846 in healthy subjects have been published[9]. Nevertheless, it is difficult to compare our data with those reference data, as the serum samples had been stored for up to 5 years. The storage time may have affected the detection of PIIANP and CS846, but the decay of the epitopes would have affected all samples equally. Bearing in mind that our data are highly exploratory, we also recognize that there are other limitations of our analysis: (1) the sample size is rather small for complex models of mutual dependency of dGEMRIC and serum biomarkers, (2) the Bonferroni method for multiple test correction may be too strict, (3) with the small sample size we may identify trends only that need to be confirmed in trials with larger sample sizes, (4) our analysis of correlations may have been affected by outliers bearing in mind that each arm only comprised 15 subjects and (5) presently, no trial exists in which the data could be replicated.

In conclusion, as our data indicate that changes of the dGEMRIC score and changes of the serum concentration of PIIANP occur in parallel, we may assume that based on these preliminary data PIIANP may be regarded as a biochemical marker which reflects anabolic changes in cartilage morphology. Moreover, we believe that PIIANP may be used as a sensitive marker in future clinical trials whose objective is to demonstrate efficacy of cartilage-regenerating compounds.

In summary, correlating imaging with chemical biomarkers in interventional or epidemiological OA trials is a field that deserves to be more profoundly explored as this approach may provide information regarding the mechanism and efficacy of agents with a disease-modifying action.

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