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Variation in Osteoarthritis Biomarkers from Activity not Food Consumption

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Abstract

Background—To optimize sampling and to understand sources of variation in biomarkers for osteoarthritis (OA), we evaluated variation due to activity and food consumption.

Methods—Twenty participants, with radiographic knee OA, provided serial serum and urine samples at 4 time points: before arising in the morning; after 1 h of light activity; 1 h after eating breakfast; and in the evening. Five serum (*s*) and 2 urinary (*u*) analytes were measured: hyaluronan (*s*HA); cartilage oligomeric matrix protein (*s*COMP); keratan sulfate (*s*KS-5D4); transforming growth factor beta (*s*TGF- β 1); and collagen II-related epitopes (*s*CPII, *u*CTX-II, and *u*C2C). Activity was monitored by an accelerometer.

Results—All serum biomarkers increased and one of the urinary biomarkers decreased after 1 h of non-exertional activity. Food consumption following activity was associated with a return of biomarker concentrations to baseline levels. Accelerometers proved to be a novel way to monitor protocol compliance and demonstrated a positive association between the mean level of activity and *s*COMP concentration. Urinary CTX-II varied the least but demonstrated both true circadian variation (peak in the morning and nadir in the evening) and the most robust correlation with radiographic knee OA.

Conclusions—We confirm activity related variation in these markers. These data suggested that biomarkers also varied due to upright posture, glomerular filtration rate stimulated by food intake, and circadian rhythm in the case of *u*CTXII.

Keywords

osteoarthritis; biomarkers; diurnal; circadian; activity

Introduction

Osteoarthritis (OA), the most common of all arthritides, is one of the most prevalent diseases and a growing health problem due to increasing longevity of the population. OA of the knee afflicts approximately 11% of people over the age of 65 y [1]. Although radiographs are commonly used to assess the severity of OA, the characteristic features of radiographic OA of joint space loss and bony remodeling represent established and significant joint damage. Radiographic endpoints in clinical trials are relatively insensitive and require large cohorts

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studied over long periods of time to provide power to detect progression. Moreover, radiographs typically quantify disease in one or only a few joints whereas OA is most often a multijoint process. Thus, it would be advantageous to have a biomarker that could accurately reflect earlier preradiographic manifestations of the disease process and sensitively quantify the overall burden of disease and rate of OA progression. Systemic biomarkers have the potential, with appropriate validation, to fulfill this need. For this purpose, it is important to understand the factors, other than disease variation, that may contribute to biomarker variation such as diurnal variation, age, gender, ethnicity, or systemic (nonrheumatologic) illnesses [2].

The general goal of this study was to investigate one source of biomarker variation, namely that related to daily or diurnal variation. We hypothesized that an improved understanding of the influences of activity and food consumption on OA-related biomarkers could facilitate the optimization of their use for clinical and clinical trial purposes. Our previous studies [3,4] of biomarker variation did not distinguish variation due to physical activity from variation due to food consumption, thus a secondary question addressed was the impact of food intake relative to activity, on biomarker variation. For at least one of these OA-related biomarkers, serum hyaluronan (sHA), significant variation (increase) related to food consumption has been demonstrated in normal volunteers [5]. It has been hypothesized that food intake stimulates clearance of pooled HA from lymphatics connected with the gastrointestinal tract, the Peyer's patches, thereby increasing serum HA concentrations. However, food intake may also stimulate glomerular filtration rate [6] and thus biomarker clearance, thereby decreasing serum biomarker concentrations. For the remaining biomarkers that we examined, the effects of food consumption are unknown. A third question examined was the correlation of intensity of activity and degree of associated variation in the biomarker. A final question evaluated was the correlation of biomarker concentrations with severity of radiographic knee OA.

Methods

Participants

Twenty participants (n=13 Female, n=15 Caucasian) with symptomatic OA of at least 1 knee (Kellgren-Lawrence grade 1–4), and without evidence of other clinically apparent arthropathies or liver disease, were invited to participate. All procedures were approved by the Institutional Review Board of Duke University. Experiments were undertaken with the understanding and written consent of each subject, and the study conformed with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964). One to two participants at a time were admitted to the Duke Clinical Research Unit (DCRU) at Duke University Medical Center for an overnight stay. No patient had received intra-articular injections of any kind for at least 6 months prior to the study. The patients were permitted to take their normal medications, which included the following (and numbers of patients consuming them): Tylenol (8); low dose aspirin (8); narcotics (2 - 1 of these Ultram); NSAIDs (9 - 3 of these on COX-2 inhibitors); nutraceuticals combination of oral glucosamine and chondroitin sulfate (4); bisphosphonates (1); thiazides (11); thyroid medication (2); statins (6). In addition, no patient was being treated with traditional rheumatoid arthritis remittive agents such as Methotrexate, Imuran, Azulfidine, or TNF-alpha inhibitors. Participants chose dinner and breakfast from a standardized menu. The breakfast items and amounts consumed were recorded. The average breakfast consumed consisted of 30.51 g protein, 60.76 g carbohydrates and 25.5 g fats.

Osteoarthritis assessment

On the day of admission, weight-bearing, fixed-flexion (30 degree) posterioranterior radiographs of both knees were taken with the SynaFlexer™ X-ray positioning frame (Synarc, San Francisco, CA) [7]. By the consensus of 2 observers (CG, VBK), knee radiographs were

scored for Kellgren-Lawrence (KL) grade [8] and individual radiographic features of OA, including joint space narrowing (JSN) and osteophytes (OST), scored semi-quantitatively (0–3 scale) using a standardized atlas [9]. Radiographic sum scores for the 2 knees were computed (sum KL, sum JSN+OST). Three patients had undergone right unilateral knee replacements and the replaced knees were assigned KL, JSN and OST scores of 0.

Blood and urine sampling

The first (of four) blood and urine specimens were taken in the evening upon admission (after dinner, between 6:00 P.M. and 8:00 P.M.) while the participant was still upright and before the participant went to bed (designated T3). The participant remained fasting past midnight and was not allowed to get out of bed after 3:00 A.M. (a bedpan or urinal was provided as necessary). In the morning, participants performed 1 h of monitored activity prior to food intake to simulate the sequence of events of the typical sampling routine in an OA clinical trial. At 8:00 A.M., while supine, the participant had a blood specimen collected and then, immediately upon arising, a first morning urine specimen was collected (designated T0). To obtain an objective measure of physical activity, a RT3 accelerometer (Stayhealthy Inc., Monrovia, CA) was placed on the participant's waist and parallel to the midline of the knee so as to most accurately measure activity in 3 dimensions with a readout in counts/min [10]. The participant then got up and dressed and performed normal morning activities for 60 min after which blood and urine samples were obtained (at 9:00 A.M. or 60 min after arising, designated T1a for T1-activity). The participant then ate breakfast while remaining seated for the next hour followed by collection of the fourth sample of blood and urine (at 10:00 A.M. or 120 min after arising, designated T1b for T1-breakfast), then was discharged home. Thus, T1a and T1b in this study corresponded to two separate aspects of the T1 timepoint analyzed in our previous study [4].

Blood and urine processing

Sera were separated and immediately frozen to -20°C within 2 h of collection, then transferred to -80°C until use. Urine specimens were centrifuged (3,500 rpm, 10 minutes) and the supernatant (top three quarters), was aliquoted for later analysis.

Quantification of OA-related biomarkers

In this study, we undertook an evaluation of 5 serum (*s*) and 2 urinary (*u*) OA-related biomarkers, associated with OA, in order to assess for the occurrence and possible causes of diurnal variability, and thus infer the optimal method and timing of sampling. The biomarkers evaluated were: hyaluronan (*sHA*); cartilage oligomeric matrix protein (*sCOMP*); keratan sulfate (*sKS-5D4*); transforming growth factor beta (*sTGF- β 1*); and collagen II-related epitopes (*sCPII*, *uCTX-II*, and *uC2C*). We chose these particular biomarkers on the basis of a previous study that demonstrated significant diurnal variation for each [3,4]. Analyses of the biomarkers were performed as previously described [4]. The serum analytes (*sHA*, *sCOMP*, *sKS-5D4*, *sTGF- β 1*, *sCPII*) were measured in ng/ml; the urine analytes (*uC2C*, *uCTX-II*) were normalized to creatinine (Cr) concentrations and reported as ng/mmol Cr.

Quantification of physical activity by accelerometer

Output files from the RT3 monitor indicate the number of “counts” recorded in each minute of the data collection period. These “counts” represent acceleration in three dimensions in arbitrary units. Using participant demographics including age, height, weight, and gender, a proprietary algorithm held by the RT3 manufacturer converts counts into kcal/min.

Statistical analysis

Statistical computations were performed using GraphPad Prism® (GraphPad Software, Inc., San Diego, CA) and JMP® (SAS, Cary, NC) software. The mean concentrations and SD of

each biomarker were calculated. Normalized biomarker concentrations were calculated to more clearly assess for diurnal variation by dividing each participant's biomarker concentration by the mean concentration of the four timepoints for that biomarker. This method accounts for individual variation among subjects. Results were analyzed using non-parametric Friedman's test with Dunn's post-hoc Multiple Comparison Test. Non-parametric Spearman's correlation analysis was used to evaluate the association of serum and urinary biomarker concentrations with radiographic OA severity (sum KL scores, and sum of JSN+OST scores for both knees). Bland Altman plots were constructed to assess for systematic bias with biomarker concentration and possible outliers [11].

Results

Twenty patients with radiographic knee OA underwent serial serum and urine sampling within a 19-h period. The ages ranged from 51 to 88 y (mean 70 ys, SD 10), and body mass index ranged from 23 to 57 kg/m² (mean 32 kg/m², SD 7). Three patients (15%) had unilateral knee replacements; six participants (30%) had unilateral knee OA, the remainder had bilateral knee OA. The sum KL scores ranged from 1 to 8 (15% sum KL-1, 30% sum KL-4, 5% sum KL-5, 15% sum KL-6, 15% sum KL-7, and 20% sum KL-8).

Activity as demonstrated by accelerometer

The mean kilocalories per minute were determined for each participant for the T0 to T1a time interval (representing the amount of energy expended during performance of activities of daily living - ADL); and the T1a to T1b time interval (representing energy expended during and after eating breakfast in a seated position). To provide a point of reference for the arthritic participants in this study, a young healthy control (CG), also wore a RT3 accelerometer to monitor physical activity for the first two hours of the morning. Just as for the participants, CG performed ADLs followed by eating breakfast while seated for an hour. The mean activity level of participants during the first hour was 1.01 kcal/min (SD 0.87). This level of activity was comparable to that of the normal healthy control performing ADLs (1.08 kcal.min). The mean activity declined from the first to second hour a mean 73%. Activity monitoring by accelerometer also provided insights into protocol compliance and demonstrated that the majority of participants complied with the instruction to be active during the first hour, and relatively sedentary (while eating) during the second hour. However, 3 of the 20 participants were deemed inactive during the first hour on the basis of low activity (<0.20 activity counts/min). Two of these individuals had bilateral severe knee OA, and one of these individuals was significantly overweight.

Effect of daily activity versus food intake on biomarker concentrations

The mean concentrations ($\pm$ SD) and range for each of the seven OA-related biomarkers at each timepoint are shown in Table 1. The median values with interquartile ranges (and maximum and minimum values) are shown in the box plots of normalized concentrations in Figure 1. The change in biomarker concentrations from T0 to T1a (effect of activity) can be compared to T1a to T1b (effect of food) in order to examine fluctuations in biomarker concentrations to determine whether activity caused a significant change in a biomarker level and the effect of food consumption following activity. To assess if the overall concentration of a biomarker influenced the extent of diurnal variation, the difference between T0, and the timepoint with greatest variation from T0, was plotted against the mean of all four timepoints. The results are graphically displayed via the Bland and Altman method in Figure 2. This method was used in order to determine if the amount of change in biomarker concentrations between two time points is a function of the amount of the biomarker. For instance might individuals with higher overall levels of a marker, vary more in relationship to activity or eating compared with individuals with low overall levels of a marker?

The normalized mean concentrations of all the serum biomarkers increased with activity (T0 to T1a) and decreased following food consumption (T1a to T1b). These changes were statistically significant for sCPII, KS-5D4, sCOMP, sHA for T0-T1a, and statistically significant for sCPII, sHA for T1a to T1b. In contrast, the mean concentration of the urinary biomarker, *u*C2C, decreased with activity and increased following food consumption, while *u*CTXII showed no significant difference due to activity or food consumption, but did show a significant decrease in the evening (T3) relative to the other timepoints. There was no association of the morning intake of fats, carbohydrates or protein with any of the biomarker results. A detailed summary of results for each biomarker is provided below and a graphical representation of the results can be seen in Figures 1 and 2.

Serum TGFβ-1 did not exhibit a statistically significant difference between T0 and T1a. However there was a statistically significant increase in concentration between T0 and T3 (increase in 17 patients) and T1b and T3 (increase in 18 patients). The magnitude of maximal variation (14% increase) was greater than the difference due to assay variability (5.3%) and was not dependent on the level of the biomarker.

Serum CPII showed a statistically significant difference between T0 and T1a (an increase in 19 patients), T1a and T1b (a decrease in 18 patients), and T1a and T3 (a decrease in 18 patients). The magnitude of maximal variation (19% increase) was greater than the difference due to assay variability (3.7%) and was not dependent on the level of the biomarker.

Serum KS-5D4 demonstrated a statistically significant difference between T0 and T1a (an increase in 19 patients), T0 and T1b (an increase in 20 patients), and T1a and T3 (a decrease in 16 patients). The magnitude of maximal variation (33% increase) was greater than the difference due to assay variability (3.7%) and was not dependent on the level of the biomarker.

Serum COMP demonstrated a statistically significant difference between T0 and T1a (an increase in 20 patients), T0 and T1b (an increase in 20 patients), and T0 and T3 (an increase in 16 patients) and T1b and T3 (a decrease in 16 patients). The magnitude of maximal variation (61% increase) was greater than the difference due to assay variability (2.5%) and was not dependent on the level of the biomarker. The level of activity in the first hour after arising, as measured by the accelerometer, correlated (R^2 0.35, $p=0.006$) with the sCOMP T1a normalized concentration (T1a/overall mean). No other biomarker was statistically significantly associated with the activity measure.

Serum HA demonstrated a statistically significant difference between T0 and T1a (an increase in 18 patients), T1a and T1b (a decrease in 16 patients), and T1a to T3 (a decrease in 17 patients). The magnitude of maximal variation (95% increase) was greater than the difference due to assay variability (<0.9%). The Bland Altman plot (Figure 2) revealed 1 outlier; when removed, the magnitude of variation was dependent (R^2 0.663, $p<0.0001$) on the level of the biomarker and was still greater than the difference due to assay variability. In addition, the amount of decline in HA with eating breakfast (and sitting) was related to overall mean level of sHA ($p=0.0005$). With the exception of sHA, there were no associations of any of the medications with any biomarker results. Serum HA was significantly lower ($p=0.02$) in statin users at T3 (64.1 ng/ml in the absence and 35.3 ng/ml in the presence of statins). In addition, the mean difference between T0 and T1a was also less ($p=0.04$) in statin users (86.5 ng/ml in the absence compared with 0.7 ng/ml in the presence of statins), although the baseline T0 level did not differ by statin intake status.

Urinary C2C demonstrated a statistically significant difference between T0 and T1a (a decrease in 15 patients), and T1a and T3 (an increase in 16 patients). The magnitude of maximal variation (27% decrease) was greater than the difference due to assay variability (2.4%) and was not dependent on the level of the biomarker.

Urinary CTXII demonstrated a statistically significant difference between T0 and T3 (a decrease in 18 patients), T1a and T3 (a decrease in 19 patients), and T1b and T3 (a decrease in 19 patients). The magnitude of maximal variation (68% decrease) was greater than the difference due to assay variability (5.9%) and was dependent (R^2 0.708, $p < 0.0001$) on the level of the biomarker (Figure 2). Even after excluding the one apparent outlier, there was still significant variability dependent on the level of *u*CTXII (R^2 0.22, $p = 0.04$). The small non-significant rise in *u*CTXII with activity (T0-T1a) was also related to mean level of *u*CTXII ($p = 0.03$),

Correlation of biomarker concentrations with severity of radiographic knee OA

We evaluated each biomarker for correlation with summed KL scores across both knees and summed JSN+OST scores. The concentrations of *u*CTXII at the T0, T1a and T1b timepoints correlated with radiographic knee OA (sum JSN+OST). The best correlation was provided by T1a and T1b (Spearman $r = 0.60$, $p < 0.01$). The *u*CTXII-T3 and sHA-T0 concentrations showed similar trends (Spearman $r = 0.4$ – 0.5 , $p = 0.09$). Only the *u*CTXII T1b concentration correlated with sum KL score (Spearman $r = 0.44$, $p = 0.05$). There were no associations of medications with radiographic knee OA status.

Discussion

Through this study we sought to further evaluate biomarker fluctuations due to physical activity and to determine whether food consumption following activity further increased serum biomarkers or decreased urinary biomarkers. Serum CPII, KS-5D4, COMP, and HA clearly demonstrated a dramatic rise after one hour of activity. All four biomarkers declined significantly thereafter; sCPII and sHA declined significantly by one hour after food intake, while KS-5D4 and COMP declined significantly by the next sampling time in the early evening. These results demonstrate that the predominant effect on the upper level of these biomarkers was exerted by the combination of physical activity and postural changes that precede food intake, causing a release of OA biomarkers into the systemic circulation, presumably from synovium, subsynovial tissue, and lymph nodes. This study confirmed diurnal variation in all of our OA-related biomarkers in a different cohort than our previous study [3,4]. Unlike *u*C2C, *u*CTXII was not effected by physical activity or food consumption. This could be explained by *u*CTXII's brief half-life and/or high clearance rate such that concentration changes would escape detection at the sampled timepoints. It is also possible that the small *u*CTXII fragments do not accumulate in the subsynovial tissues of the joint and/or Peyer's patches as do for instance, hyaluronan fragments, thus showing little variation with joint movement and food consumption. Moreover, of these markers, only *u*CTXII demonstrated diurnal variation confined to late in the day. This suggests that *u*CTXII may be much less susceptible to effects of timing and activity related variation early in the day and therefore may offer an advantage in the context of clinical trials incorporating morning body fluid sampling.

The synchronous diurnal variation in sTGF β , sCPII, and sKS-5D4 throughout the day in active and inactive participants (not shown) suggested an effect of posture alone on biomarker variation. Blood volume is typically 600–700 ml less in an adult in an upright compared with a recumbent position [12]. This is caused by loss of protein-free fluid through the capillaries and results in a 10% reduction in blood volume and an 8–10% increase in plasma protein concentrations. This order of magnitude variation was consistent with the first hour variation in sTGF β and sCPII for both the normalized biomarker concentrations and the mean absolute biomarker concentrations. The first hour variation in normalized biomarker concentrations for sKS-5D4 were greater than 10% and are likely due to a combination of postural effects and clearance of this biomarker from the joint due to activity. The decline in the urinary marker, *u*C2C, within the first hour of arising was compatible with the known postural effects on renal

protein filtering; namely, by 1 hour after assuming an upright posture, both renal blood flow and glomerular filtration rate decrease causing decreased urine production and decreased urine protein excretion in most individuals [6,12–14]. Our previous work [4] revealed that the decline in μ CTXII was delayed (occurred at 4 hours after arising) relative to μ C2C and therefore likely represents variation on the basis of something other than postural-related variation. We have found that μ CTXII is highly correlated to urinary bone resorption markers (unpublished data). The urinary bone resorption marker, uNTXI, has been shown to vary in a circadian pattern with a peak in the morning and a nadir in the afternoon [15]; the diurnal variation of CTXII is entirely consistent with that of NTXI and therefore most likely represents true circadian variation as opposed to variation on the basis of activity, posture or food. With respect to the other markers, following food consumption, their concentrations reverted toward baseline values. This meant they declined in serum and increased in urine. The opposite effects seen between serum collagen and urinary collagen markers are most compatible with stimulation of glomerular filtration rate by food intake.

Serum HA was the only biomarker in this study showing an association with statin use. There were no associations of the other biomarkers with any of the other medications. The rise in sHA with activity was less in the statin users, and the late day level (T3) was also significantly lower in the statin users, although the morning recumbent (T0) concentration did not differ between groups. Serum HA is considered to be a marker of joint inflammation and the majority of synovial fluid HA is produced by the fibroblast-like synoviocytes lining the joint. Hyaluronan depolymerization by reactive oxygen species at sites of inflammation generates hyaluronan fragments [16] that are cleared from the subsynovial tissues with joint use and activity. Statins have anti-inflammatory properties outside their lipid lowering actions. Statins *in vitro* at pharmacological doses, are known to inhibit production of IL-6 and IL-8 by fibroblast-like synoviocytes and to inhibit proliferation of these cells in response to TNF- α [17]. The HA results suggest a joint tissue anti-inflammatory effect of statins in keeping with *in vitro* findings of anti-inflammatory effects; however, intriguing as these observation are, they require confirmation in an independent and larger cohort.

A previous study by Andriacchi showed that levels of sCOMP increased when measured 30 min after exercising [18]; interestingly, we found this to be the one biomarker whose relative increase at 1 h after arising was correlated with amount of activity by accelerometer. Quantifying activity by accelerometer also proved to be very useful for objectively monitoring compliance with physical activity instructions. Two of the three relatively inactive participants had bilateral knee OA (sum KL scores of 8) and the third had the highest BMI at 56 kg/m². The use of accelerometers to document activity prior to body fluid sampling for clinical trials may be a useful method of understanding inherent variability of results, particularly in the case of sCOMP.

Our previous assessment of these biomarkers demonstrated that samples obtained in the afternoon and evening yielded the strongest correlations with radiographic severity of knee OA and morning stiffness [4]. Although not necessarily powered to evaluate an association of biomarkers with radiographic OA, we observed an association of μ CTXII concentrations at T0, T1a and T1b with radiographic features of OA (sum JSN+OST). The robustness of correlation offers an advantage for clinical trials in which time of sampling might not be able to be stringently controlled. These data also underscore the potential for convenient blood sampling to provide clinically useful biomarker data.

There were several limitations of this study. First, it involved a small sample size that nevertheless confirmed morning activity related variation of 6 of the 7 biomarkers evaluated in our previous study. The time interval, 1 hour between the various body fluid sampling, may not have captured the maximal magnitude of the diurnal variation. Second, we do believe that

postural changes play a role in the rise in serum and the decrease in urinary (C2C) levels. However, we only measured the effect of food consumption after activity as opposed to the effect of food consumption on its own. This was done for the practical reason that patients in clinical trials or seeing specialists typically perform activity prior to traveling to the clinic. We therefore do not know what the effects of food consumption alone would have been. Finally, this study primarily addressed biomarker variation related to morning activity and the impact of food intake following morning activity.

In summary, OA biomarker concentrations varied diurnally in association with physical activity while food consumption after activity did not further increase serum, or decrease urinary, biomarker concentrations. The timepoint with greatest variation, the fasting samples obtained one hour after arising, (T1a), corresponds to the timepoint when blood is typically obtained for biomarker studies in a clinical trial. In contrast, the hour after eating food was associated with a decline in the serum biomarkers and an increase in the marker uC_2C . Ultimately, larger studies will be crucial to determine which one or group of biomarkers are best able to predict OA disease activity and progression. The ongoing validation of OA-related biomarkers provides a growing armamentarium of potential adjunctive outcome measures in clinical trials of disease modifying medical interventions for OA, of growing concern as the epidemic of OA is continuing to increase both in the U.S. and worldwide.

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List of Abbreviations

OA	osteoarthritis
(sHA)	Hyaluronan in serum
sCOMP	cartilage oligomeric matrix protein in serum
sKS-5D4	keratan sulfate in serum
sTGF-β1	transforming growth factor beta in serum
sCPII	collagen II propeptide in serum
uCTX-II	C-telopeptide of Collagen II in urine
uC2C	carboxy-terminal cleavage neoepitope of Collagen II in urine
(s)	serum

(u)	urinary
KL	Kellgren Lawrence grade of knee osteoarthritis severity
ADLs	activities of daily living
JSN	joint space narrowing
OST	osteophyte

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Figure 1. Box Plots of Normalized Biomarker Concentrations at Different Timepoints

Normalized biomarker concentrations were derived for each participant by dividing a biomarker concentration by the mean concentration of the four timepoints for that biomarker. Box and whisker plots demonstrate the median, 25th and 75th percentiles (box), and minimum and maximum values (whiskers); *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. ADL (activities of daily living) occurred between timepoints T0 and T1a ('T1 activity'), and FOOD intake occurred between timepoints T1a and T1b ('T1 breakfast').

Figure 2. Bland Altman Plots for each Biomarker

These plots were constructed by calculating the difference between T0 and the timepoint with greatest variation from T0, and plotting this difference against the mean of all four timepoints. The timepoint with greatest variation for each biomarker is listed on the y axis. Only serum HA and urinary CTXII showed more dramatic diurnal variation with higher mean serum (HA) or urine (CTXII) concentrations of the biomarker.

Table 1

Mean biomarker concentrations at measured time interval.

Biomarker	Timepoint			
	T0	T1a	T1b	T3
sTGF- β 1 ng/ml	29 $\pm$ 8 (17–467)	31 $\pm$ 9 (14–50)	27 $\pm$ 7 (16–45)	33 $\pm$ 10 (167–57)
sCPII ng/ml	833 $\pm$ 193 (460–1189)	992 $\pm$ 266 (540–1673)	808 $\pm$ 160 (484–1128)	828 $\pm$ 170 (461–1172)
sKS-5D4–5D4 ng/ml	1513 $\pm$ 542 (554–3106)	2007 $\pm$ 659 (1076–3449)	1803 $\pm$ 596 (914–3332)	1708 $\pm$ 631 (728–3299)
sCOMP ng/ml	1163 $\pm$ 453 (619–2199)	1870 $\pm$ 546 (994–2970)	1613 $\pm$ 615 (834–2801)	1369 $\pm$ 556 (682–2613)
sHA ng/ml	59 $\pm$ 74 (4–323)	115 $\pm$ 102 (12–387)	57 $\pm$ 42 (13–181)	54 $\pm$ 39 (9–155)
uC2C ng/mmol Cr	2426 $\pm$ 1509 (963–6468)	1761 $\pm$ 937 (900–4781)	2475 $\pm$ 2155 (907–10600)	2545 $\pm$ 1443 (533–5947)
uCTXII ng/mmol Cr	379 $\pm$ 329 (125–1568)	404 $\pm$ 369 (107–1826)	415 $\pm$ 367 (127–1869)	258 $\pm$ 207 (34–940)

Values shown are the mean $\pm$ SD (range) concentrations for each timepoint for each of the biomarkers for 20 participants (s=serum, u=urinary).