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An Autologous Protein Solution prepared from the blood of osteoarthritic patients contains an enhanced profile of anti-inflammatory cytokines and anabolic growth factors

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Abstract

The objective of this clinical study was to test if blood from osteoarthritis (OA) patients (n = 105) could be processed by a device system to form an autologous protein solution (APS) with preferentially increased concentrations of anti-inflammatory cytokines compared to inflammatory cytokines. To address this objective, APS was prepared from patients exhibiting radiographic evidence of knee OA. Patient metrics were collected including: demographic information, medical history, medication records, and Knee Injury and Osteoarthritis Outcome Score (KOOS) surveys. Cytokine and growth factor concentrations in whole blood and APS were measured using enzyme-linked immunosorbent assays. Statistical analyses were used to identify relationships between OA patient metrics and cytokines. The results of this study indicated that anti-inflammatory cytokines were preferentially increased compared to inflammatory cytokines in APS from 98% of OA patients. APS contained high concentrations of anti-inflammatory proteins including 39,000 ± 20,000 pg/ml IL-1ra, 21,000 ± 5,000 pg/ml sIL-1RII, 2,100 ± 570 pg/ml sTNF-RI, and 4,200 ± 1,500 pg/ml sTNF-RII. Analysis of the 82 patient metrics indicated that no single patient metric was strongly correlated ($R^2 > .7$) with the key cytokine concentrations in APS. Therefore, APS can be prepared from a broad range of OA patients.

Keywords

IRAP; IL-1; TNFα; platelet-rich plasma; APS; Pain

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Introduction

Osteoarthritis (OA) is a debilitating disease, and there is currently no common treatment that prevents or inhibits its progression. The inflammatory cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) have been shown to play a critical role driving the progression of OA[1]. These cytokines can cause both pain [2] and cartilage degeneration [3]. Antagonists of IL-1β or TNFα, such as recombinant IL-1 receptor antagonist (IL-1ra) or the soluble receptor for TNFα (sTNF-R), have been explored independently as OA therapies [4] but have not yet been proven efficacious [5]. Therefore, OA therapies that inhibit multiple inflammatory signaling pathways may be required to address the limitations of currently available therapies.

Autologous blood-derived products have been investigated as a possible therapy to treat OA because they contain molecules that target multiple signaling pathways. An autologous protein solution (APS) has been developed which is composed of: 1) white blood cells (WBCs) containing anti-inflammatory proteins, 2) platelets containing anabolic growth factors, and 3) concentrated plasma which contains anti-inflammatory proteins and anabolic growth factors [3,6-8]. This combination of WBCs, platelets, and concentrated plasma has produced solutions with increased concentrations of anti-inflammatory cytokines and anabolic growth factors from control donors [3]. Treatment with APS has demonstrated anti-inflammatory and chondroprotective effects in preclinical cell culture [6,7] and explant testing and decreased lameness in horses with naturally occurring OA in a prospective randomized clinical trial [8]. These positive tissue culture and animal clinical trial results support further evaluation of APS as a potential therapy for OA, beginning with the characterization of APS produced from blood taken from OA patients.

Research on autologous products has motivated the need for an autologous product containing the components of APS. Previously, platelet-rich plasma (PRP) intra-articular injections have been investigated as a treatment for osteoarthritis [9]. Surrounding these studies, there has been debate regarding whether or not WBCs should be included in the autologous therapies [10]. However, *in vitro* experimentation [11], preclinical animal [12], and clinical testing in humans [13] have demonstrated that WBCs produce and mediate the production of anti-inflammatory cytokines. For example, WBC-containing PRP (termed L-PRP [14]) reduced the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a primary mediator of the inflammatory process, in cultured articular chondrocytes challenged with TNFα [11]. In an equine trial, L-PRP significantly decreased lameness and joint effusion [12]. In humans, L-PRP treatment was safe and resulted in a greater clinical improvement in OA symptoms than hyaluronic acid [15]. Taken together, these studies suggest that autologous products containing WBCs may play a role in modulating inflammation and should be further explored as a potential treatment for OA.

In this study, we hypothesized that the concentration of anti-inflammatory cytokines were increased over inflammatory cytokines in APS from OA patients. To test this hypothesis we compared cytokine profiles of APS and blood from either patients with diagnosed OA or control donors. Also, the possible effects of OA patient demographics, comorbidities, and concomitant medications on these profiles were explored.

Materials and Methods

OA patients (n = 105) were enrolled (NCT01050894) according to an IRB-approved protocol at four sites (University of Kentucky: IRB# 09-0785-F3R, Ohio State University: IRB study # 1113947, OrthoIndy/Orthopedics Research Foundation:

St. Francis Project # 652, Orthopedic Sports Medicine Center, Elkhart Indiana: IRB study # 1113947). The sample size was selected to account for OA patients with diverse comorbidities, concomitant medications, survey scores, and OA indicators. Inclusion in the study required radiographic evidence of knee OA including joint space narrowing (JSN), osteophytes, subchondral sclerosis, or subchondral cysts. Patients were excluded from the study if they were pregnant or less than 18 years of age. Medical conditions that excluded patients were as follows: hemophilia or other blood clotting disorders, active hematologic cancer, currently undergoing chemotherapy, history of rheumatoid arthritis, septic joint, fracture, active infection or history of chronic infection. Patients who had used cytokine-blocking drugs in the previous 6 months were also excluded. Patients were required to sign an informed consent form prior to inclusion in the study and subsequently filled out Knee injury and Osteoarthritis Outcome Surveys (KOOS). KOOS is a subjective survey which contains five categories of questions about perception of affected knee pain within the past week including symptom sum (KOOSSS), pain (KOOSP), function- daily living (KOOSFDL), function- sports and recreation (KOOSFSR), and quality of life (KOOSQOL) [16]. A list of comorbidities and concomitant medications were also acquired from each patient (Supplementary Figure 2). Control donor samples were collected during internal testing studies at Biomet (WIRB # 1115097).

From each patient, 54 ml of whole blood was drawn with an 18-gauge apheresis needle into a 60 ml syringe containing 6 ml anticoagulant citrate dextrose solution, formula A (ACD-A, Citra Labs, Braintree, MA). Baseline blood was also drawn into a syringe containing ACD-A at a ratio of 1 to 9. To prepare APS, blood from the 60 ml syringe was transferred to the APS Separator (Biomet Biologics, Warsaw, IN). The device was processed using a centrifuge (Drucker Company, Philipsburg, PA) at 3200 rpm (1800g) for 15 minutes. The cell solution was then extracted and transferred to an APS Concentrator (Biomet Biologics, Warsaw, IN). The device was processed, and approximately 2-3 ml of APS was removed from the device. No platelet activation agents were combined with APS in this study. Baseline blood and APS were transferred to 15 ml centrifuge tubes labeled with patient number, patient initials, time and date in preparation for shipment.

For cytokine analysis, samples from three of the sites were shipped in dry ice. Samples from the fourth site were transported on the date of processing. Those samples were immediately frozen post-transportation. All samples were stored in a freezer at -50°C. Each sample was thawed once and aliquoted to enable the enzyme-linked immunosorbent assays (Quantikine ELISA kits, R&D Systems, Minneapolis, MN) which contain cell membrane lysis reagents to release cytokines and growth factors. The concentrations of cytokines and growth factors were characterized in the baseline blood and APS of each of the 105 patient samples (measured proteins included: TNFα, IL-6, IL-8, IL-1β, sTNF-RI, sTNF-RII, IL-1ra, sIL-1RII, epidermal growth factor (EGF), insulin like growth factor-1 (IGF-1), platelet

derived growth factor-AB (PDGF-AB), PDGF-BB, and transforming growth factor-β1 (TGF-β1). Patient medical and medication history was used to identify any comorbidities or concomitant medications that may affect the APS concentrations of these cytokines from OA patients. Key cytokine and growth factor concentrations from control donors were determined from samples from normal subjects (Western IRB Study # 1115097).

According to a Kolmogorov-Smirnov Test for Normality, most cytokine and growth factor profiles did not meet the normality assumption required for a Pearson R-squared analysis of correlation. For this reason, a nonparametric Spearman Rank correlation ($\alpha = 0.05$) was performed to determine significant univariate associations between APS cytokines, whole blood cytokine concentration, concomitant diseases, medications, and KOOS scores. A stepwise multiple regression analysis of the interactions was performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC). The univariate markers were examined for confounding effects, and stratification and stepwise linear regression were used to determine the driver variables in the relationships. Important interactions and their corresponding p-values were reported.

Results

Patient demographics demonstrated the distribution of radiographic evidence of OA including joint space narrowing, osteophytes, subchondral sclerosis, or subchondral cysts (Table 1). Patients were enrolled in a sequential manner. A total of 9 patients were enrolled at the University of Kentucky, 34 patients were enrolled at Ohio State University, 8 patients were enrolled at OrthoIndy, and 54 patients were enrolled at the Orthopedic Sports Medicine Center. Six blood samples were excluded from cytokine analysis due to protocol deviations which would affect measured cytokine concentrations, including blood draw errors such as inadequate ACD-A volume or incorrect blood draw volume, preventing proper blood processing (n = 3). A device processing error resulted in an insufficient APS sample volume (n = 1), and shipment errors resulted in sample thawing and subsequent clotting (n = 2). Excluding these samples still enabled the analysis of cytokines and growth factors from (n = 99) donors. Demographic information on control donors is included in Supplementary Table 1.

Cytokine analyses of whole blood and APS indicated a wide range of cytokines available to be delivered as a potential treatment for OA (Table 2). Anti-inflammatory cytokines were significantly concentrated in the APS compared to whole blood (2.2- 5.9 fold) ($p < 0.05$ for all cytokines tested) (Table 2). Inflammatory cytokines were also concentrated in the APS compared to whole blood ($p < 0.05$ for all cytokines tested) but at lower average fold increases than measured for their corresponding anti-inflammatory counterparts. For example, while 42,000 ± 20,000 pg/ml IL-1ra was detected in APS, only 8.9 ± 7.3 pg/ml IL-1β was found, representing a IL-1ra:IL-1β ratio of 5,900 ± 2,900 (Table 2). Additionally, the concentrations of anabolic growth factors, including PDGF-BB, IGF-1, EGF, and TGF-β1, were increased in the APS compared to their concentrations in whole blood ($p < 0.05$ for each growth factor tested). The concentration of PDGF-AB in whole blood and APS was not significantly different ($p = 0.0864$) (Table 2).

APS contained an improved ratio of anti-inflammatory cytokines to inflammatory cytokines compared to their baseline ratio in whole blood. Of the devices, 98% tested had an improved Combined Anti-Inflammatory Cytokine Blocking Ratio (Equation 1 and Table 3).

Combined Anti-Inflammatory Cytokine Blocking Ratio

$$\frac{\frac{[IL-1ra+sIL-1RII]}{IL-1\beta}APS}{\frac{[IL-1ra+sIL-1RII]}{IL-1\beta}Blood} or \frac{\frac{[sTNF-RI+sTNF-RII]}{TNF\alpha}APS}{\frac{[sTNF-RI+sTNF-RII]}{TNF\alpha}Blood} > 1.0 \quad \text{Equation 1}$$

Similarly, 91% of the devices had improved Anti-IL-1 Cytokine Blocking Ratios (Equation 2). Also, 90% of the devices had improved Anti-TNFα Cytokine Blocking Ratios (Equation 3)

Anti-IL-1 Cytokine Blocking Ratio

$$\frac{\frac{[IL-1ra+sIL-1RII]}{IL-1\beta}APS}{\frac{[IL-1ra+sIL-1RII]}{IL-1\beta}Blood} > 1.0 \quad \text{Equation 2}$$

Anti-TNFα Cytokine Blocking Ratio

$$\frac{\frac{[sTNF-RI+sTNF-RII]}{TNF\alpha}APS}{\frac{[sTNF-RI+sTNF-RII]}{TNF\alpha}Blood} > 1.0 \quad \text{Equation 3}$$

The fold increase in key anti-inflammatory cytokines and anabolic growth factors, whose recombinant counterparts have been tested as treatments for OA, were similar in APS from OA and control donors (Table 4). For example, control donors' APS contained an average of 6.6 fold greater concentration of IL-1ra than baseline blood; whereas OA patients' APS contained an average of 5.9 fold greater concentration of IL-1ra than baseline blood. The fold increase of inflammatory IL-1β was 3× and 2.8× for control donors and OA patients, respectively. Anabolic IGF-1 was similarly concentrated 1.5× for both control donors and OA patients.

Regression analyses were used to detect if there were any relationships between patient metrics (37 disease categories, 36 medications, five KOOS subsections, and four OA indicators). There were no strong Spearman Rank correlations ($R^2 > 0.70$) [17] found between any cytokines, comorbidities, concomitant medications, OA indictors, or KOOS scores. There were several significant but weak correlations (R^2 of 0.30 to 0.70) between anti-inflammatory cytokine concentrations in APS and concomitant diseases, medications, OA indicators, and KOOS scores (Table 5).

Discussion

The results of this study provide evidence that the APS device system preferentially increases anti-inflammatory cytokines over inflammatory cytokines. APS contained

similarly concentrated cytokines and growth factors from control donors and OA patients. Regression analysis indicated that of the 37 disease categories, 36 medications, five KOOS subsections, and four OA indicators recorded for each patient (Supplementary Figure 2), no single patient metric exhibited a strong positive or negative correlation with the key anti-inflammatory and inflammatory cytokines in APS. Although there has been research exploring the relationship between obesity and inflammatory cytokine concentration in baseline whole blood [18], there was no correlation between obesity and the cytokine profile of processed APS. The high concentrations of anti-inflammatory cytokines and anabolic growth factors can be attributed to the components of APS which include WBCs, platelets, and plasma.

The high concentrations of IL-1ra, a key anti-inflammatory protein in APS, can be attributed to WBCs. WBCs have been identified as the primary source of IL-1ra in human tissues [19]. APS contains WBCs, whereas the output of commercially available devices that produce conditioned serums do not contain WBCs [20,21]. The output of these devices contains only serum prepared from blood incubated with glass beads for 6 - 24 hours. These extended incubation periods with glass beads also induce the production of IL-1and TNFα in horses [22] and humans [23]. It has been published that an IL-1ra:IL-1 ratio (pg/ml IL-1ra / pg/ml IL-1) of at least 1000 is necessary to inhibit IL-1[24]. In donor-paired experiments, APS contained greater concentrations of anti-inflammatory cytokines (Il-1ra, sIL-1RII, sTNF-RI, and sTNF-RII) and anabolic growth factors (PDGF-AB, PDGF-BB, TGF-β, and EGF), and lower concentrations of inflammatory cytokines (IL-1β and TNFα) compared to autologous conditioned serums [25]. The presence of cells in APS may allow for the continued production of IL-1ra, sIL-1RII, sTNF-RI, and sTNF-RII after injection, which is not possible with cell-free serums. Also, conditioned serums are typically frozen after their extended incubation period for later injections [26], which can lead to cytokine degradation [27]. The presence of cells and never-frozen concentrated plasma allows APS to deliver high concentrations of bioactive anti-inflammatory cytokines and anabolic growth factors.

The concentrated plasma in APS also contains molecules which may promote cartilage healing and inhibit inflammation. IGF-1 is predominantly secreted into the plasma by the liver [28]. IGF-1 induces stem cell differentiation down the chondrogenic lineage and cartilage extracellular matrix deposition [29]. Fibrin clots loaded with supraphysiologic concentrations of IGF-1 led to increased cartilage healing in a large-defect equine model, but not complete tissue repair [30]. sIL-1RII is a plasma-residing form of the IL-1 receptor which has been cleaved from the cell surface. This soluble receptor binds and inhibits IL-1 in solution with high affinity due to the molecules' slow dissociation rate [31]. sTNF-RI and sTNF-RII are soluble forms of TNF-RI and TNF-RII which are cleaved from the cell surface, bind TNFα, and reside in the plasma [32]. The presence of multiple anti-inflammatory cytokines in the concentrated plasma and WBCs has enabled APS to inhibit both IL-1 and TNFα in cartilage explant testing using blood from control donors [7].

In addition to WBCs and plasma, APS contains platelets which contain important growth factors that may play a role in reducing inflammation associated with OA. Platelets contain alpha granules which store PDGF, EGF, and TGF-β [33]. Anabolic growth factors from platelets may also have pleiotropic effects on repairing tissue damage from OA. For

example, PDGF promotes production of collagen by chondrocytes which is essential for proper cartilage function and regeneration [34]. PDGF in combination with IGF-1 has also been shown to decrease IL-1-mediated NF-κB activation and cartilage degradation [35]. EGF has been shown to stimulate chondrocyte proliferation [36] and increase the responsiveness of chondrocytes to IGF-1[37]. Short-term treatments with TGF-β have increased chondrogenesis and blocked inflammation by exerting immunosuppressive effects on lymphocytes [38]. In this study, no platelet activating agents were used to maintain consistency with previous bench top and large animal APS testing. In a separate study, growth factors were released from platelets in PRP when combined with synovial fluid without an exogenous platelet activator. Addition of thrombin to PRP increased the concentrations of TNFα and IL-6 in synovial fluid in vivo [39]. Together, the anabolic growth factors from platelets in APS may play an essential role in tissue repair and inhibition of inflammation. Taken together, available evidence suggests that an ideal candidate for study as an OA treatment should contain: 1) WBC-derived anti-inflammatory proteins, 2) platelet-derived growth factors, and 2) plasma-associated growth factors and cytokines.

The approach to forming an intermediate concentrated cell solution could have had a significant role in determining the composition of APS and its ability to inhibit inflammation. Commercially available systems that produce concentrated solutions without WBCs (Leukocyte-Reduced PRP) have not demonstrated high platelet recoveries, which are necessary to obtain significant concentrations of anabolic growth factors [40]. High platelet recoveries are not possible in commercial systems without forming a buffy-coat, which contains both WBCs and platelets. Further concentrating the WBCs and platelets from the intermediate cell solution is also critical to inhibit inflammatory signaling. For example, APS and its concentrated solution of anti-inflammatory cytokines was more effective than the intermediate cell solution at inhibiting MMP-13 production by IL-1β- and TNFα-stimulated chondrocytes [41]. The improved inhibition of MMP-13 production was attributed to the higher concentrations of IL-1ra, sTNF-RI, and sTNF-RII in APS compared to the intermediate cell solution.

Conclusions

The current study provides the first evidence that anti-inflammatory cytokines and growth factors could be preferentially concentrated in APS from OA patients. APS from control donors and OA donors contained similar concentrations of anti-inflammatory cytokines and anabolic growth factors. These results, in combination with previous *in vitro* cell culture studies, tissue explants studies, and an equine clinical trial provide compelling evidence that APS is a promising candidate for investigation as a treatment for OA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Demographic information of 105 OA patients. Data reported as mean ± standard deviation range.

Parameter	**Finding**				
Gender	Male	Female			
	50.5%	49.5%			
Age	58 ± 11 (22-85)				
BMI	31.7 ±7.6 (20.2-61.1)				
Race	Caucasian	African American	Hispanic	Asian	Other
	92%	3%	3%	1%	1%
Smokers	13%				
KOOS: Symptoms and Stiffness	52.4 ±19.4				
KOOS: Pain	49.4 ±17.9				
KOOS: Daily Living	55.6 ±18.9				
KOOS: Sports and Rec	27.5 ±23.8				
KOOS: Quality of Life	32.7 ±19.3				
KOOS: Total	48.7 ±17.7 (2.4-96.4)				
Osteophytes	77%				
Subchondral Sclerosis	75%				
Subchondral Cysts	28%				

Table 2

Cytokine profile in whole blood and APS from OA patients. ND = non-detectible concentration of cytokine using ELISA assays. NA = not available due to non-detectible concentration of cytokine in whole blood.

	Cytokine	Concentration in Whole Blood (pg / ml)	Concentration in APS (pg / ml)	Average Fold Increase	p Value
Anti-inflammatory Cytokines	**IL-1ra**	7,600 ± 2,500	42,000 ± 20,000	5.9	0.015
	sIL-1RII	9,500 ± 2,500	21,000 ± 6,300	2.2	<0.0001
	sTNF-RI	810 ± 280	3,000 ± 960	3.9	<0.0001
	sTNF-RII	1,500 ± 490	5,100 ± 1,900	3.5	<0.0001
Inflammatory Cytokines	**IL=1β**	3.3 ±1.1	8.9 ± 7.3	2.8	0.0245
	IL-8	74 ±30	290 ± 190	4.2	0.0013
	IL-6	1.8 ±1.3	3.0 ± 3.5	1.6	<0.0001
	TNF-α	ND	4.3 ± 3.0	NA	NA
Anabolic Growth Factors	**PDGF-AB**	17,000 ±5,700	38,000 ± 25,000	2.5	0.0864
	PDGF-BB	5,300 ± 2,400	12,000 ± 8,700	2.5	0.0006
	IGF-1	79,000 ± 22,000	120,000 ± 43,000	1.5	<0.0001
	EGF	370 ± 200	710 ± 490	2.2	0.0031
	TGF-β1	57,000 ± 57,000	150,000 ± 150,000	4.2	<0.0001

Table 3

Percent of devices that processed blood from OA donors to form APS with improved (> 1.0) Anti-IL-1 Cytokine Blocking ratios ([IL-1ra + sIL-1RII / IL-1β]APS / [IL-1ra + sIL-1RII / IL-1β]Blood), Anti-TNFα Cytokine Blocking Ratio ([sTNF-RI + sTNF-RII / TNFα]APS / [sTNF-RI + sTNF-RII / TNFα]Blood), and Combined Anti-Inflammatory Cytokine Blocking Ratio (([IL-1ra + sIL-1RII / IL-1β]APS / [IL-1ra + sIL-1RII / IL-1β]Blood) or ([sTNF-RI + sTNF-RII / TNFα]APS / [sTNF-RI + sTNF-RII / TNFα]Blood)).

Ratio	% of Devices with Improved Cytokine Blocking Ratios
Anti-IL-1 Cytokine Blocking Ratio	90.8%
Anti-TNFα Cytokine Blocking Ratio	89.8%
Combined Anti-Inflammatory Cytokine Blocking Ratio	98.0%

Table 4

Comparison of key cytokines and growth factors in APS from OA patients and control donors. ND = nondetectible concentration of cytokine using ELISA assays. NA = not available due to non-detectible concentration of cytokine in whole blood.

Cytokine		Baseline Concentration	APS Concentration (pg/ml)	Fold Increase
IL-1ra	Control (n = 92)	6,300 ± 3,000	39,000 ± 20,000	6.6
	OA (n = 99)	7,600 ± 2,500	42,000 ± 20,000	5.9
sIL-IRII	Control (n = 51)	10,000 ± 2,500	21,000 ± 5,000	2.1
	OA (n = 99)	9,500 ± 2,500	21,000 ± 6,300	2.2
sTNF-RI	Control (n = 53)	650 ± 490	2,100 ± 570	3.7
	OA (n = 99)	810 ± 280	3,000 ± 960	3.9
sTNF-RII	Control (n = 64)	1,000 ± 360	4,200 ± 1,500	4
	OA (n = 99)	1,500 ± 490	5,100 ± 1,900	3.5
IL-1β	Control (n = 56)	2.7 ± 1.2	7.2 ± 3.1	3
	OA (n = 99)	3.3 ± 1.1	8.9 ± 7.3	2.8
TNFα	Control (n = 26)	ND	1.7 ± 0.8	NA
	OA (n = 85)	ND	4.3 ± 3.0	NA
IGF-1	Control (n = 48)	34,000 ± 37,000	52,000 ± 60,000	1.5
	OA (n = 98)	79,000 ± 22,000	120,000 ± 43,000	1.5

Table 5

Comorbidities, concomitant medications, OA indicators, and KOOS scores that weakly correlated (R2 of 0.30 to 0.70) with the concentration of anti-inflammatory cytokines in APS.

	Metric
Weakly Positively Correlated with > 1 Anti-inflammatory Cytokine	Hypertension, Age
Weakly Negatively Correlated with > 1 Anti-inflammatory Cytokine	None
Weakly Positively Correlated with > 1 Inflammatory Cytokine	None
Weakly Negatively Correlated with > 1 Inflammatory Cytokine	Hypertension
Weakly Positively Correlated with > 1 Anabolic Growth Factor	KOOS FSR
Weakly Negatively Correlated with > 1 Anabolic Growth Factor	Proton Pump Inhibitors
% of Metrics Weakly Correlated with Cytokine or Growth Factor Concentration	6.1%
% of Metrics Strongly Correlated with Cytokine or Growth Factor Concentration	0%