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Circulating Fibroblast Activation Protein and Dipeptidyl Peptidase 4 in Rheumatoid Arthritis and Systemic Sclerosis

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Abstract

Objectives—To quantify circulating fibroblast activation protein (cFAP) and dipeptidyl peptidase 4 (cDPP4) protease activities in patients with rheumatoid arthritis (RA), systemic sclerosis (SSc), and a control group with mechanical back pain and to correlate plasma levels with disease characteristics.

Methods—Plasma was collected from patients with RA (n=73), SSc (n=37) and control subjects (n=26). DPP4 and FAP were quantified using specific enzyme activity assays.

Results—Median cDPP4 was significantly lower in the RA group (p=0.02), and SSc group (p=0.002) compared with controls. There were no significant differences in median cFAP between the three groups. DPP4 and FAP demonstrated a negative correlation with inflammatory markers and duration of disease. There were no associations with disease subtypes in RA, including seropositive and erosive disease. Decreased cDPP4 was found in SSc patients with myositis. Plasma FAP was lower in RA patients receiving prednisone (p=0.001) or leflunomide (p=0.04), but higher with biologic agents (p=0.01). RA patients receiving leflunomide also had decreased cDPP4 (p=0.014). SSc patients receiving prednisone (p=0.02) had lower cDPP4 but there was no association with cFAP.

Conclusions—No association was found between cFAP and RA or SSc. Plasma DPP4 was decreased in RA and SSc when compared with controls. cDPP4 and cFAP correlated negatively

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Author CONTRIBUTIONS

Design experiment, collect data, analyse data, write paper: PS, WL, NM, MG
Generate data, analyse data: AR
Project design, provision of know-how: WB
Data provision, data interpretation: HE, GH, DS

Potential Conflicts of Interest

Professor William W. Bachovchin is a director of Arisaph Pharmaceuticals Inc., which provided the FAP-specific substrate 3144-AMC.

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with inflammatory markers and there were no significant correlations with disease characteristics in this RA cohort.

Keywords

proteases; biomarkers; inflammation; enzymes; fibroblasts; lymphocytes

Introduction

Dipeptidyl peptidase 4 (DPP4; DPP-IV) is a multifunctional cell surface glycoprotein, also known as CD26 or adenosine deaminase binding protein. It is expressed by a wide range of cell types, primarily lymphocytes, endothelial cells and epithelial cells. DPP4 has a rare peptidase activity that preferentially cleaves proline or alanine dipeptides from the N-terminal of polypeptides, often altering their bioactivity. Its substrates include chemokines and neuropeptides, such as stromal cell-derived factor-1 (SDF-1; CXCL12), CXCL10, RANTES (CCL5), neuropeptide Y and substance P. DPP4 binds adenosine deaminase, promotes T cell proliferation and provides a costimulatory signal for T cell activation [1–3].

Fibroblast activation protein (FAP) is closely related to DPP4 and has a similar protease function. In contrast to DPP4, it is expressed only by activated fibroblasts and some macrophages in sites of tissue remodelling and wound healing [4–8]. In addition to DPP4 - like activity, it possesses a post-proline endopeptidase activity that exerts a gelatinase activity [9–11], inactivates fibroblast growth factor (FGF)-21 [12, 13], processes alpha-2 antiplasmin into a more active form [11], and has a role in the invasion of cells in collagenous matrices [14].

DPP4 and FAP also circulate in soluble form in blood [1, 15–18]. DPP4 and FAP are each measured by a specific, direct enzymatic assay [19–23], but FAP has also been measured in antibody-based assays such as ELISA [9, 24]. Previous studies have shown decreased levels of circulating DPP4 (cDPP4) in patients with rheumatoid arthritis (RA), systemic lupus erythematosus, systemic sclerosis (SSc) and inflammatory bowel disease, and following acute ischaemic stroke, compared with controls[2325]. Similarly, expression of DPP4 is lower in RA synovial fluid, compared with osteoarthritis synovial fluid [26]. Conversely, T cell surface expression of DPP4 in blood is increased in RA compared with controls [27]. Circulating FAP (cFAP) levels have been shown to vary in two other disease states. In patients with acute coronary syndromes, levels were lower than controls [28, 29]. Conversely, both intrahepatic FAP and cFAP are elevated in patients with cirrhosis [19, 24] and in many patients with severe fibrosis [20, 30]. Circulating levels of cFAP has not been investigated previously in RA or SSc.

Increased expression of FAP has been demonstrated on RA synovial fibroblasts (RASF) [31]. It has been hypothesised that RASF has a key role in the pathogenesis of RA, having the potential to migrate between joints, leading to destruction of previously unaffected cartilage [32]. It is possible that FAP has a role in this process.

The roles of DPP4 and FAP in autoimmune and inflammatory disease are unclear. The primary aim of this study was to use validated, specific, direct enzymatic assays [19–22] to compare plasma levels of cFAP and cDPP4 in patients with RA, SSc, and a control group

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with mechanical back pain. The secondary outcomes of interest were the correlations between cFAP and cDPP4 levels and disease characteristics.

Materials and Methods

The research protocol for this project was approved by the Western Sydney Local Health District Human Research Ethics Committee and conforms to the provisions of the World Medical Association's Declaration of Helsinki. Informed consent was obtained from all participants. Patients were recruited from inpatients and outpatient clinics at Westmead Hospital and from private rheumatology consulting rooms. Plasma was collected in EDTA tubes from patients with RA, SSc and control subjects with mechanical back pain. Mechanical back pain was the chosen control because such patients were similar to the subjects regarding demographic parameters. Patients were excluded if they were under 18 years of age, had a history of malignancy, or were unable to provide written consent. Samples were separated by centrifugation at 1500 rpm for 10 minutes, and stored at −80°C. Routine clinical biochemistry and immunology blood tests were performed by commercial pathology laboratories, including liver and renal function tests, erythrocyte sedimentation rate (ESR), C-reactive protein (CPR), rheumatoid factor (RF), anti-cyclic citrullinated peptide (CCP) antibodies, antinuclear antibodies (ANA) and antibodies to extractable nuclear antigens (ENA).

DPP4 Enzyme Assay

The DPP4 assay using the protease substrate H-Gly-Pro-p-nitroaniline (pNA) (Sigma-Aldrich, St Louis, MO, USA) and chromogenic standards in a linear range of 0–60 nmol of pNA was as recently described [19, 21]. Briefly, triplicate plasma samples of 10 μl were incubated at 37°C and read at 405 nm in a FluoStar plate reader (BMG Labtech, Germany). Three in-house controls were used to normalise the final data. One unit of DPP4 activity produces 1.0 micromole of pNA per minute. The intra-assay CV was 3.5 ± 2.5 % and the inter-assay CV was 4.2 ± 3.5 %.

DPP4 contributes 95–98% of the hydrolysis of H-Gly-Pro-pNA by plasma or serum [15, 19, 22, 33]. The source of the residual 2–5% hydrolysis of H-Gly-Pro based substrates is unknown, but is unlikely to be DPP8, DPP9, FAP, DPP7 or prolyl endopeptidase [15, 33].

FAP Enzyme Assay

Plasma levels of FAP were measured using an in-house enzyme activity assay that has been described and validated recently [19, 20]. The validation included establishing specificity by showing that the substrate is not hydrolyzed by plasma or tissue sourced from the FAP-negative mouse or by plasma from a FAP-negative human [19]. The FAP-specific substrate 3144-AMC, duplicate fluorescent standards in a linear range of 0–600 pmol of amino methylcourmarin (AMC) and triplicate plasma samples of 5 μl of a 1/5 dilution in PBS were incubated at 37°C then read in a Fluostar plate reader at excitation 355 nm and emission 450 nm. Three in-house controls in each assay and were used to normalise the final data. The intra-assay coefficient of variation (CV) is 6.2 ± 3.5% and the inter-assay CV is 19.7 ± 8.35%.

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Statistical Analyses

Data were analysed using parametric and nonparametric statistical methods using IBM SPSS version 21. Mann-Whitney tests were used for group comparisons and Spearman rank correlations to assess relationships between parameters. P values below 0.05 were considered statistically significant.

Results

73 patients with RA, 37 with SSc and 26 control subjects were recruited for the study (Table 1). The groups were similar in terms of age and comorbidities, but there was a female predominance in the RA and SSc groups compared with the control group. Median (interquartile range; IQR) plasma DPP4 activity was significantly less in RA (21.2 U/L (16.2–27.5 U/L), p=0.02) and SSc (18.6 U/L (15.6–22.7 U/L), p=0.002) than control (26.14 U/L (21.0–29.7 U/L)) (Fig. 1). The difference in DPP4 activity between RA and SSc was not statistically significant (p=0.22). There was no significant difference in median (IQR) plasma FAP activity between the control group (1077.3 pmol AMC/min/mL (955.5–1363.5 pmol AMC/min/mL)) and RA (1009.5 pmol AMC/min/mL (755.8–1443.2 pmol AMC/min/mL), p=0.10) or SSc (1186.4 pmol AMC/min/mL (917.2–1575.3 pmol AMC/min/mL), p=0.86) (Fig. 2). The cDPP4 and cFAP levels were comparable to previously published data [19, 34].

Secondary analyses are exploratory and as such are presented to inform future studies and explore potential associations. Plasma cDPP4 and cFAP were independent of gender across all groups (p=0.58 and p=0.78 respectively). Osteoporosis was more common in the RA group. The presence of diabetes or liver disease may affect DPP4 and FAP levels [19, 24, 35, 36]. However, there was no significant difference in the frequency of abnormal liver function tests or diabetes between the groups. Furthermore, the presence of abnormal liver function tests was not associated with any significant differences in DPP4 (p=0.61) or FAP (p=0.08) across all patients. The presence of renal impairment (eGFR<60 mL/min/1.73m^2) was not associated with any difference in DPP4 (p=0.77) or FAP (p=0.10) activity.

Rheumatoid Arthritis

In secondary outcome analyses for patients with RA, cDPP4 and cFAP enzyme activity levels were compared with disease characteristics, including RF and CCP antibody status, joint erosions and the presence of nodules. There were no statistically significant differences between the groups (Table 2). When cDPP4 and cFAP were correlated with current treatment, patients receiving prednisone or leflunomide had lower levels of cFAP (p= 0.01) and cDPP4 (p=0.04). There were too few patients receiving gold therapy to perform a meaningful comparison. cFAP was reduced in patients receiving biologic agents (p= 0.01). These included anti-TNF agents such as etanercept, infliximab and adalimumab, as well as anti-IL6 therapy (tocilizumab), anti-CD20 antibody (rituximab) and T cell costimulation modulator (abatacept) (Table 2). There were also some significant correlations with markers of disease activity. There was a negative correlation between the inflammatory markers, ESR and CRP, and cDPP4 and cFAP levels. There was no association with joint count, but there

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was a weak negative correlation with duration of disease, defined as the number of years since diagnosis (Table 3).

Systemic Sclerosis

In the secondary analyses, plasma DPP4 and FAP levels were compared with disease characteristics in SSc. There was a lower level of cDPP4 in patients with myositis (p=0.03). There were no statistically significant correlations found with other disease characteristics (Table 4).

Similarly to the RA group, there was a negative correlation between inflammatory markers and cDPP4 and cFAP. There was no association with disease duration (Table 3). Decreased cDPP4 levels were found in patients receiving prednisone treatment (Table 4).

Discussion

This study is the first investigation of cFAP in RA and SSc. Our data did not demonstrate any significant difference in cFAP levels in patients with RA or SSc versus controls. Consistent with the published literature on a small cohort of 35 patients [27], our data demonstrates that cDPP4 levels are decreased in patients with RA and SSc, compared with controls. The enzyme activity levels of DPP4 and FAP were measured.

There were negative correlations of cDPP4 and cFAP enzyme activities with inflammatory markers. In previous reports, some studies have shown a similar negative correlation [37], whereas one has reported no correlation with CRP or ESR [25]. There are some differences between that latter study cohort and the patients included in our study. Their patients had shorter median disease duration (5.5 years versus 13 years), and in our study we demonstrated a weak negative correlation between cDPP4 and disease duration. Furthermore, Ulusoy et al excluded patients receiving leflunomide or biologic agents [25]. All patients in that study were receiving corticosteroids with a mean prednisolone dose of 7.5 mg/day [25], as compared with 47.9% in our cohort. Our data indicate that treatment may affect levels of cDPP4 and cFAP; this may have affected the overall association seen in our data as all of our RA patients were receiving treatment, often with combination therapy.

Tamaki et al have previously reported decreased levels of soluble DPP4 in a cohort of 56 patients with SSc compared with controls [38], which is in keeping with our results. However, they also found significantly decreased levels in patients with diffuse cutaneous disease compared with limited cutaneous disease and a negative association with the modified Rodnan's total skin thickness scores [38]. In our cohort of 37 SSc patients, median cDPP4 levels appeared to be greater in patients with limited cutaneous disease (20.0 U/L) vs diffuse cutaneous disease (14. 9U/L), but this difference was not statistically significant (p=0.06). Decreased expression of DPP4 has been demonstrated on skin biopsy and dermal fibroblasts from patients with SSc compared with controls [39], and it might therefore be expected that patients with more extensive skin disease could have lower cDPP4 levels.

Although there are several hypotheses concerning the decrease in circulating protease levels in inflammatory diseases, the source of cDPP4 is not known [40]. It has been suggested that

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the process of shedding DPP4 from the cell surface is inhibited in inflammation [40]. This could account for the observation that the number of lymphocytes expressing DPP4 is increased in RA, whereas plasma levels are decreased [41]. DPP4 may play a dual role in RA and inflammatory disease. On one hand, its peptidase activity inactivates important cytokines and neuropeptides such as substance P and SDF-1, which are responsible for promoting recruitment and retention of activated T cells within inflamed joints. Experiments in murine models have shown that inhibition of DPP4 leads to increased invasion of RASF into cartilage. In vitro, inhibition of the peptidase activity of DPP4 and FAP can lead to increased levels of active forms of the chemokines CXCL10 and CXCL12 (SDF-1) [4243]. However, DPP4 expression on T cells is strongly upregulated in RA and it is thought to act as a T cell co-stimulatory molecule [3], and, in a very large human cohort, lower risk of RA has been associated with DPP4 inhibition [44]. It may be that the bound and soluble forms of DPP4 have differing roles in inflammation.

In this first investigation of cFAP in RA and SSc, no significant differences in cFAP levels were detected. This may be related to the more restricted expression of FAP compared with DPP4, as FAP is found only on activated fibroblasts and circulating levels may therefore show little variation in RA or SSc, despite increased expression by RASF. By contrast, cFAP is increased in patients with liver cirrhosis [19, 24]. This may be a reflection of the relatively large size of the liver compared with joint tissue, such that upregulation of FAP in the chronically fibrotic liver leads to an increase in circulating levels that is sufficiently large to be detected in plasma. Concordant with our data here, cFAP remains low in patients with arterial thrombosis [29] and in patients with steatosis and little or no liver fibrosis [20].

Our data indicated a weak positive correlation between plasma DPP4 and FAP levels (Spearman rank correlation coefficient = 0.390; $p<0.001$). As the two enzymes are expressed by different cell types, a reason for this correlation is unclear. The levels of both proteases drop with inflammation, but the mechanism for this is unknown.

Although larger than a concordant previous study [27], our sample size was a limitation. It is possible that some differences in disease subtypes were not detected as the sample was too small to reach statistical significance. Another limitation was that not all of the patients with RA had radiographs performed at the time of sample collection. In these cases, the presence or absence of erosions was determined by examining the medical records and previous imaging studies. It is therefore possible that in some patients cDPP4 and cFAP levels might have changed since the imaging occurred. In the context of intense efforts to construct disease biomarker panels, this study importantly adds to the understanding of which human diseases can alter cDPP4 and cFAP levels.

Conclusion

Plasma levels of DPP4 were decreased in RA and SSc when compared with controls., whereas cFAP was not associated with either RA or SSc. Both cDPP4 and cFAP demonstrated a negative correlation with inflammatory markers. There were no significant correlations between cDPP4 or cFAP and disease characteristics in RA, but some trends were seen with disease subtypes in SSc. The roles of DPP4 and FAP in inflammatory disease

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are not well understood, and further research is required to understand their mechanism of action and potential as biomarkers of disease.

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Abbreviations

cDPP4	Circulating dipeptidyl peptidase 4
cFAP	circulating fibroblast activation protein
RA	rheumatoid arthritis
SSc	systemic sclerosis
SDF-1	stromal cell-derived factor-1
CXCL	chemokine ligand
RASF	rheumatoid arthritis synovial fibroblasts
EDTA	ethylenediaminetetraacetic acid
ESR	erythrocyte sedimentation rate
CPR	C-reactive protein
RF	rheumatoid factor
CCP	anti-cyclic citrullinated peptide
ANA	antibodies; antinuclear antibodies
ENA	extractable nuclear antigens
pNA	p-nitroaniline
AMC	amino methylcoumarin
CV	coefficient of variation
IQR	interquartile range
eGFR	estimated glomerular filtration rate
DMARD	disease modifying anti-rheumatic drug

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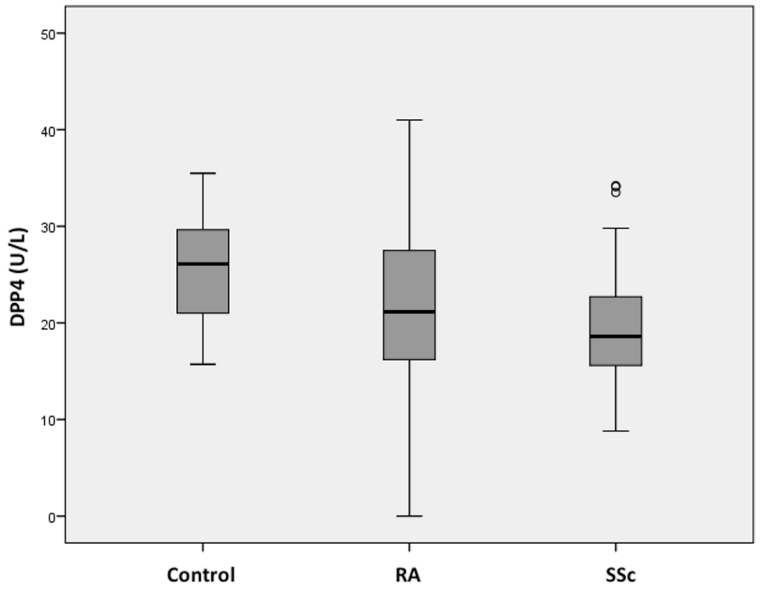

Figure 1. Circulating DPP4 in Control, RA and SSc Patients

Box plot displaying plasma DPP4 levels in RA and SSc patients, compared with controls. There are significantly decreased levels in RA (n=73; p=0.02) and SSc patients (n=37; p=0.002).

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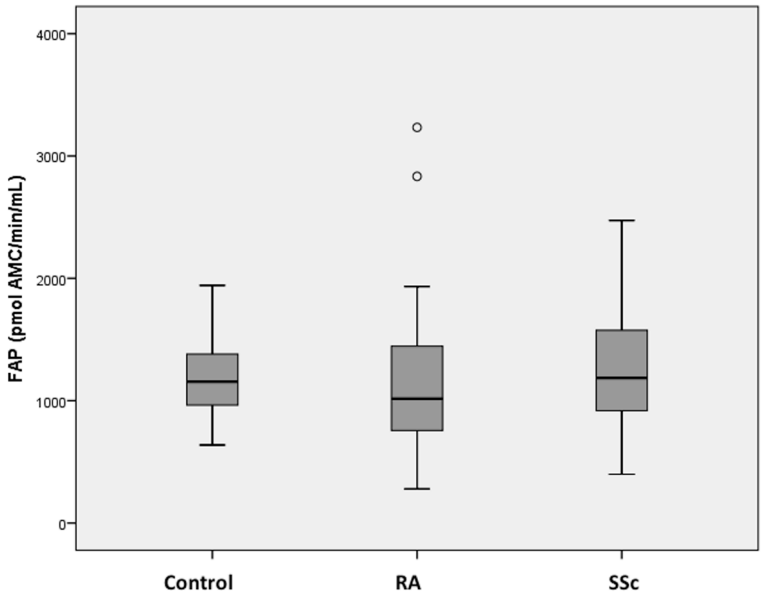

Figure 2. Circulating FAP in Control, RA and SSc Patients

Box plot displaying plasma FAP levels in RA (n=73) and SSc (n=37) patients, compared with controls (n=26). There are no significant differences between the groups.

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Table 1

Baseline Demographics

	Rheumatoid Arthritis	Systemic Sclerosis	Back Pain
N	73	37	26
Median age - years (interquartile range)	63.0 (53.0–68.0)	54.0 (49.0–64.0)	57.5 (41.0–66.0)
Median time since diagnosis – years (interquartile range)	13.0 (5.5–22.5)	5.0 (3.0–10.0)	-
Gender - female	52 (71%)	30 (81%)	11 (42%)
Abnormal liver function tests	11 (15%)	7 (19%)	8 (31%)
Impaired renal function (eGFR<60mL/min/1.73m^2)	7 (10%)	3 (8%)	5 (19%)
Diabetes	8 (11%)	1 (3%)	4 (15%)
Osteoporosis	26 (36%)	6 (16%)	4 (15%)
Hypertension	30 (41%)	9 (24%)	5 (19%)
Rheumatoid factor positive	54 (74%)	-	-
Anti-CCP antibody positive	50 (72%)†	-	-
Erosions on X-ray	46 (67%)†	-	-
Rheumatoid nodules	20 (27%)	-	-
Anti-Scl70 antibody positive	-	15 (11%)	-
Anti-centromere antibody positive	-	7 (5%)	-
Anti-RNA polymerase III antibody positive	-	4 (3%)	-
Limited cutaneous	-	19 (53%)‡	-
Joint involvement	-	14 (62%)	-
Raynaud's phenomenon	-	37 (100%)	-
Digital ulcers	-	18 (49%)	-
Pulmonary fibrosis	-	20 (54%)	-
Pulmonary hypertension	-	11 (30%)	-
Renal involvement	-	4 (11%)	-
Muscle disease	-	9 (24%)	-
Prednisone	35 (48%)	10 (27%)	-
Methotrexate	53 (73%)	5 (14%)	-
Leflunomide	13 (18%)	0	-
Sulfasalazine	14 (19%)	0	-
Hydroxychloroquine	12 (16%)	0	-
Gold	3 (4%)	0	-
Biologic disease modifying anti-rheumatic drug (DMARD)	36 (49%)	0	-
Cyclosporin	1 (1%)	3 (10%)	-
Mycophenolate	0	5 (14%)	-
Intravenous immunoglobulin	0	2 (6%)‡	-
Cyclophosphamide	0	6 (17%)‡	-

†N=69

[≠]N=36

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Table 2

Subgroup Analysis in RA

	Plasma Level Median (IQR)			
Disease Characteristic and number of subjects	**DPP4 (U/L)**	**P**	**FAP (pmol AMC/min/mL)**	**P**
Rheumatoid factor		0.48		0.11
54 Positive	21.3 (9.4)		1037.9 (668.9)	
19 Negative	20.8 (12.3)		788.7 (705.7)	
Anti-CCP antibody		0.27		0.18
50 Positive	20.9 (10.4)		1037.4 (688.9)	
19 Negative	21.5 (10.7)		842.5 (518.4)	
Erosions on X-ray		0.82		0.21
46 Present	21.2 (9.8)		1051.5 (685.5)	
23 Absent	21.1 (13.6)		913.4 (506.3)	
Rheumatoid nodules		0.33		0.50
20 Present	20.9 (8.8)		1013.2 (666.7)	
53 Absent	21.4 (12.6)		1037.4 (747.4)	
Treatment and number				
Prednisone		0.08		0.001
35 Yes	20.0 (10.6)		826.7 (455.4)	
38 No	21.5 (12.0)		1167.7 (627.1)	
Methotrexate		0.51		0.39
53 Yes	20.9 (7.2)		1018.8 (689.6)	
20 No	23.8 (17.9)		849.7 (796.0)	
Leflunomide		0.01		0.04
13 Yes	16.2 (8.2)		838.2 (310.5)	
60 No	21.5 (10.6)		1051.2 (701.9)	
Sulfasalazine		0.61		0.27
14 Yes	20.9 (6.6)		1085.8 (580.3)	
59 No	21.2 (13.6)		984.9 (741.1)	
Hydroxychloroquine		0.21		0.72
12 Yes	20.4 (11.3)		1101.9 (745.6)	
61 No	21.2 (12.2)		989.8 (700.7)	
Biologic DMARD		0.08		0.01
36 Yes	22.6 (12.9)		1081.3 (678.1)	
37 No	20.7 (10.3)		895.3 (734.7)	

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Table 3

Markers of Disease Activity

		Rheumatoid Arthritis			
		ESR	**Duration of CRP**	**Disease**	**Tender/swollen Joint Count**
DPP4	**Correlation coefficient**	−0.322	−0.429	−0.247	0.016
	P	0.03	<0.001	0.04	0.90
FAP	**Correlation coefficient**	−0.426	−0.352	−0.058	0.123
	P	0.003	0.002	0.63	0.31
		Systemic Sclerosis			
DPP4	**Correlation coefficient**	−0.454	−0.288	−0.233	-
	P	0.039	0.110	0.116	
FAP	**Correlation coefficient**	−0.412	−0.323	−0.137	-
	P	<0.001	0.001	0.15	

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Table 4

Subgroup Analysis in SSc

	Plasma Level Median (IQR)			
Disease Characteristic and number of subjects	**DPP4 (U/L)**	**P**	**FAP (pmol AMC/min/mL)**	**P**
Antibody status		0.11		0.97
7 Anti-centromere positive	24.5 (13.5)		1218.8 (825.3)	
15 Anti-Scl70 positive	18.6 (15.9)		1192.4 (713.7)	
Cutaneous involvement		0.06		0.35
19 Limited	20.0 (6.7)		1186.4 (648.4)	
18 Diffuse	14.9 (13.7)		1192.4 (917.3)	
Joint involvement		0.06		0.53
14 Present	16.1 (7.6)		1047.4 (1074.6)	
23 Absent	20.8 (9.7)		1218.8 (600.5)	
Digital ulcers		0.26		0.41
18 Present	18.6 (8.7)		1079.1 (887.7)	
19 Absent	20.6 (11.3)		1269.9 (645.7)	
Pulmonary fibrosis		0.36		0.78
20 Present	18.6 (9.8)		1189.4 (800.9)	
17 Absent	20.0 (9.6)		1044.1 (741.9)	
Pulmonary hypertension		0.50		0.23
11 Present	19.3 (12.8)		1114.0 (602.7)	
26 Absent	18.6 (10.0)		1189.4 (780.9)	
Renal involvement		0.38		0.35
4 Present	16.4 (16.0)		1445.8 (1255.5)	
33 Absent	19.3 (7.9)		1150.3 (738.2)	
Muscle disease		0.03		0.24
9 Present	13.3 (9.2)		1728.9 (1021.5)	
28 Absent	20.3 (10.2)		1132.2 (591.4)	
Treatment				
Prednisone		0.02		0.09
10 Yes	13.2 (7.9)		819.9 (1093.0)	
27 No	20.6 (10.5)		1218.8 (594.5)	
Methotrexate		0.42		0.11
5 Yes	17.0 (10.8)		1761.9 (1473.3)	
32 No	19.7 (9.0)		1168.4 (746.8)	
Mycophenolate		0.35		0.48
5 Yes	21.6 (16.3)		917.2 (834.7)	
32 No	18.5 (7.8)		1192.4 (658.4)	
Cyclophosphamide		0.25		0.33
6 Yes	14.8 (11.7)		1097.2 (675.1)	
30 No	19.7 (9.5)		1244.4 (725.0)	