ORIGINAL ARTICLE

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Plasma-Soluble Biomarkers for Fibrodysplasia Ossificans Progressiva (FOP) Reflect Acute and Chronic Inflammatory States

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ABSTRACT

Fibrodysplasia ossificans progressiva (FOP) is a progressive, debilitating genetic disease in which skeletal muscle and connective tissue is episodically replaced by heterotopic bone. Discovery of surrogate biomarkers of disease (genotype)-related and flare-up-associated activity of FOP in a readily accessible matrix, such as plasma, would facilitate an understanding of the complex pathophysiology of FOP, aid patient care, and provide a valuable tool for the development and monitoring of potential therapeutics. In a case–control study, using a carefully collected and curated set of plasma samples from 40 FOP patients with the classic ACVR1^{R206H} mutation and 40 age- and sex-matched controls, we report the identification of disease-related and flare-up-associated biomarkers of FOP using a multiplex analysis of 113 plasma-soluble analytes. Adiponectin (implicated in hypoxia, inflammation, and heterotopic ossification) as well as tenascin-C (an endogenous activator of innate immune signaling through the TLR4 pathway and a substrate for kallikrein-7) were highly correlated with FOP genotype, while kallikrein-7 was highly correlated with acute flare-up status. Plasma-soluble biomarkers for FOP support a flare-up-related acute inflammatory phase of disease activity superimposed on a genotypic background of chronic inflammation. © 2021 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: FIBRODYSPLASIA OSSIFICANS PROGRESSIVA; HETEROTOPIC OSSIFICATION; BIOMARKERS; ADIPONECTIN; TENASCIN-C; KALLIKREIN-7

Introduction

Fibrodysplasia ossificans progressiva (FOP; MIM#135100) is an ultra-rare, genetic disorder of heterotopic ossification (HO) that results in progressive joint ankylosis, ultimately rendering movement impossible.[1-3] FOP may progress insidiously but most often episodically, heralded by flare-ups—spontaneous or trauma-associated inflammatory events—characterized variably by swelling, pain, redness, joint stiffness, and/or loss of mobility and leading to clinical and radiographic HO.[3]

FOP is caused by a recurrent heterozygous activating mutation in activin receptor A type I (ACVR1), a bone morphogenetic protein (BMP) type I receptor, in all classically affected individuals (617G>A; R206H).[4] Currently, diagnosis of FOP is confirmed by mutation analysis, not by any single or combination of biomarkers. In vitro and in vivo evidence from human and mouse studies in FOP strongly suggests that the ACVR1^{R206H} mutation confers a chronic pro-inflammatory state independent of acute flare-ups.[5-10]

There have been limited published studies on the discovery of biomarkers in FOP.[11,12] Tissue biopsy and evaluation of lesional biomarkers would exacerbate the disease and therefore is not clinically feasible.[2] Progress toward identifying biomarkers has been impeded by the ultra-rare nature of the condition, difficulty with sample collection due to the burden and potential for harm in the collection process, as well as the logistics of timely and accurate sample collection with respect to disease activity.

Discovery of surrogate biomarkers of disease (genotype)-related and flare-up-associated activity of FOP in a readily accessible matrix, such as plasma, would facilitate an understanding of the complex pathophysiology of FOP and would be helpful for patient care and for the development and monitoring of potential therapeutics. Using a carefully collected and curated set of

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plasma samples, we identified potential FOP biomarkers using multiplex analysis of 113 plasma-soluble analytes.

Materials and Methods

Patients and sample collection

Blood samples were collected from FOP patients and their family members as part of routine clinical care visits in the Department of Orthopaedic Surgery at the Perelman School of Medicine of the University of Pennsylvania. Blood was collected in 10 mL K2 EDTA tubes (BD [Franklin Lakes, NJ, USA] cat. #366643) for adults and children older than 2 years or 4 mL K2 EDTA tubes (BD cat. #367861) for children younger than 2 years of age. Samples were maintained at room temperature until processing 2 to 24 hours later.

Preparation of samples

Plasma was separated by one of two methods, depending on whether peripheral blood mononuclear cells (PBMCs) were isolated from the samples for other reasons. Undiluted blood was layered over Ficoll-Paque (GE Healthcare [Chicago, IL, USA] cat. #17-1440-02) in one to two 15 mL conical tubes, and centrifuged at room temperature for 20 minutes at 800*g* with no brake. Plasma was collected from the top layer (leaving behind PBMCs, red blood cells, and Ficoll) and then transferred to a new 15 mL tube. The plasma was centrifuged for 10 minutes at 1400*g* with brake to remove residual cells, and the supernatant was aliquoted into 1.5 mL cryovials. Completed samples were stored at or below −80°C. Alternatively, undiluted blood was centrifuged in the original collection tube at 1400*g* for 20 minutes at room temperature. Plasma was transferred to a new 15 mL tube and centrifuged for 10 minutes at 1400*g* to remove residual cells. The resulting supernatant was aliquoted into 1.5 mL cryovials and stored at or below −80°C.

One cryovial for each chosen sample was thawed on ice, mixed briefly by flicking the tube, and then 500 μL aliquots were distributed into 1.5 mL screw-top microfuge tubes, which had been prelabeled and chilled. These were flash-frozen in a dry-ice/ethanol bath and then organized into freezer boxes. These were stored at −80°C and transported by overnight shipment on dry ice to Myriad Rules Based Medicine (RBM; Salt Lake City, UT, USA) for multiplex analysis.

Clinical determination of flare-up status

We measured the levels of 113 analytes in plasma samples from four subject groups: unaffected individuals and individuals with FOP by flare-up status (active, remote, quiescent). Flare-up status was arbitrarily defined by the time from appearance of symptoms and signs clinically determined to be consistent with the last episodic exacerbation at the time of sample collection. Flare-up status was defined as following: (i) an *active* flare-up is a current/ongoing clinical flare-up at the time of sample collection; (ii) a flare-up is *remote* when it has occurred within 1 to 2 years of sample collection; and (iii) *quiescent* status occurs when the last flare-up occurred more than 2 years before sample collection.

Multiplex Luminex analysis

Multiplex Luminex analysis was performed by Myriad RBM. All samples were stored at less than −80°C until tested. Samples were thawed at room temperature, vortexed, spun at 3700*g* for 5 minutes for clarification, and transferred to a master microtiter plate. Using automated pipetting, an aliquot of each sample was added to individual microsphere multiplexes of the selected Multi Analyte Profile and blocker. This mixture was thoroughly mixed and incubated at room temperature for 1 hour. Multiplexed cocktails of biotinylated reporter antibodies were added robotically and, after thorough mixing, incubated for an additional hour at room temperature. Multiplexes were labeled using an excess of streptavidin-phycoerythrin solution, thoroughly mixed and incubated for 1 hour at room temperature. The volume of each multiplexed reaction was reduced by vacuum filtration and washed three times. After the final wash, the volume was increased by addition of buffer for analysis using a Luminex instrument and the resulting data interpreted using proprietary software developed by Myriad RBM.

For each multiplex, both calibrators and controls were included on each microtiter plate. Eight-point calibrators to form a standard curve were run in the first and last column of each plate and controls at three concentration levels were run in duplicate. Standard curve, control, and sample QC were performed to ensure proper assay performance. Study sample values for each of the analytes were determined using four- and five-parameter logistics, with weighted and non-weighted curve fitting algorithms included in the data analysis package.

IBM-Watson for drug discovery (IBM-WDD)

IBM-WDD was used to generate relationship networks from biomarker predictions. Biological relationship network extraction was applied to proteins significantly associated with FOP genotype and flare-up status based on previously described methods for analysis of pathway interactions between differentially expressed proteins.[13] The confidence was set to >95%, and captured links were supported by at least two published documents.

Study design and statistics

This was a case–control study. Forty patient samples, with age- and sex-matched controls to the extent possible, were stratified on the basis of flare-up status at the time of sample collection. There were 113 biomarkers investigated in the analysis. For any biomarker, if more than 50% of assessment values were either below the lower limit of quantification (LLOQ), or below the lower limit detection (LDD), not readable (NR), or above the upper limit of quantification (ULOQ), the biomarker was not analyzed in any models. No imputation was performed. Adjustment for age (ie, <15 years and ≥15 years) was based on ≥95% skeletal maturity.[14]

Two-way ANOVA model

To determine whether there was any significant difference for a given biomarker between FOP subjects and control subjects, a two-way ANOVA model was fit for the individual biomarker assessment value adjusted for FOP genotype and age group. A p value <0.05 for FOP genotype was considered statistically significant.

To assess a possible relationship between a biomarker and flare-up status, FOP subjects were grouped into two categories, active disease and inactive disease. The FOP active group included subjects who had current/ongoing clinically-confirmed flare-ups at the time of sample collection. The FOP inactive group included subjects whose last flare-up was at least 1 or more years

before the time of sample collection (ie, remote and quiescent disease status as defined above). Similarly, to investigate whether there was any significant difference in a given biomarker between the FOP active group and FOP inactive group, a two-way ANOVA model was fit for each biomarker assessment adjusted by FOP flare-up status and age group. Only FOP subjects were included in this model. A p value <0.05 was considered statistically significant.

Logistic regression model

A logistic regression model was conducted for predicting FOP genotype adjusted by individual biomarker assessment and age group. If the p value was <0.05 for biomarker assessment, the biomarker was considered as a statistically significant predictor of FOP genotype (ie, could distinguish between FOP subjects versus control subjects) for a given age group.

Similarly, a logistic regression model was also built for predicting FOP flare-up status adjusted by individual biomarker assessment and age group. If the p value was <0.05 for the biomarker assessment, the biomarker was considered as a statistically significant predictor for FOP flare-up status (ie, could distinguish between active versus inactive disease state) for a given age group.

Hierarchical clustering analysis (HCA)

A hierarchical clustering algorithm was applied to organize biomarkers, FOP genotype, and flare-up status variables into groups or clusters. First, all variables were treated as clusters (initially of size 1) and similarity distances between all pairs of clusters were calculated. The closest pair of clusters were merged, the similarity distances were updated, and the process repeated until only one cluster remained, which contained all of the original variables. The sequence of merges is presented as a dendrogram. Variables that share a common link near the "branches" of the dendrogram (right side) were merged earlier and are considered more closely related to those that are linked near the "root" of the dendrogram (left side).

Study approval

Collection of samples was approved by the Institutional Review Board of the University of Pennsylvania and written informed consent was received before inclusion in the study.

Results

Patient age and flare-up status

We measured the levels of 113 analytes in plasma samples (Supplemental Table S1) from four subject groups: unaffected individuals and individuals with FOP with known flare-up status (active, remote, quiescent). The age and flare-up status of the FOP patients from whom the samples were obtained are shown in Table 1. Control samples (ie, from individuals without FOP) were obtained from 17 males (age range 6 to 32 years) and 23 females (age range 2 to 31 years).

Table 1. Demographics of Sample Donors

Age (years)	Sex	Flare-up status
1.78	F	Active
2.12	F	Active
2.18	M	Active
3.99	F	Active
3.21	M	Active
5.49	M	Active
5.20	F	Active
6.34	M	Active
6.56	F	Active
8.50	M	Active
9.02	F	Active
10.43	F	Active
10.79	F	Active
16.22	F	Active
17.96	F	Active
20.36	M	Active
23.73	F	Active
23.77	F	Active
24.48	F	Active
28.11	F	Active
0.6	F	Quiescent
6.12	F	Remote
7.13	F	Remote
8.68	F	Remote
9.09	F	Remote
11.69	M	Remote
12.33	F	Quiescent
13.72	F	Quiescent
15.79	M	Quiescent
16.81	F	Quiescent
18.37	F	Remote
19.91	F	Remote
20.15	M	Quiescent
22.72	F	Remote
25.57	M	Quiescent
25.40	M	Quiescent
26.28	M	Quiescent
28.19	F	Quiescent
28.24	M	Quiescent
31.38	M	Quiescent

F = female; M = male.

Soluble biomarkers altered by classic FOP genotype ($ACVR1^{R206H}$)

The biomarkers with the most significant results by ANOVA or logistical regression related to genotype are shown in Table 2. Although 18 analytes showed relationships with respect to FOP genotype, after adjustment for age, adiponectin and tenascin-C displayed the highest level of significance across the analyses. Fig. 1 shows the distribution of adiponectin and tenascin-C plasma levels between individuals with FOP and unaffected individuals.

As an alternative method to evaluate biomarkers related to genotype, hierarchical clustering analysis (HCA) was used. The dendrogram in Fig. 2 shows that analytes most similar to the FOP genotype variable are myoglobin (MB) and tumor necrosis factor receptor 2 (TNFR2).

Soluble biomarkers altered by FOP flare-up status

For analyses of biomarkers that were altered by disease activity or flare-up status, individuals with remote and quiescent activity were combined into a single group with inactive status. For each analyte, flare-up status (active versus inactive) was evaluated by ANOVA and logistical regression (Table 3), respectively.

Table 2. Biomarkers Associated With Fibrodysplasia Ossificans Progressiva (FOP) Genotype

Biomarker	Model (FOP[a] versus control, $n = 80$)			
	ANOVA (p value)	ANOVA [age group adjusted] (p value)	Logistical regression (p value)	Logistical regression [age group adjusted] (p value)
Adiponectin (ADIPOQ)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Alpha-1-antitrypsin (AAT)	**0.0366**	**0.0202**	**0.0497**	**0.0342**
Alpha-2-macroglobulin (A2M)	**0.0278**	0.4106	**0.0324**	0.4050
Apolipoprotein(a) (Lp(a))	**0.0269**	0.0990	**0.0335**	0.1055
Collagen IV	**0.0280**	0.1166	**0.0327**	0.1173
Ferritin (FRTN)	0.0945	**0.0097**	0.1049	**0.0185**
Insulin-like growth factor-binding protein 2 (IGFBP-2)	**0.0004**	**0.0011**	**0.0015**	**0.0037**
Insulin-like growth factor-binding protein 7 (IGFBP-7)	**0.0077**	**0.0278**	**0.0136**	**0.0329**
Kallikrein-7 (KLK-7)	**0.0119**	**0.0191**	**0.0209**	**0.0269**
Leptin (LEP)	**0.0445**	0.1345	0.0631	0.1500
Macrophage inflammatory protein-1 beta (MIP-1 β)	**0.0327**	0.0860	**0.0407**	0.0865
Matrix metalloproteinase-3 (MMP-3)	0.2454	**0.0099**	0.2632	**0.0369**
Osteocalcin	**0.0399**	0.6415	**0.0458**	0.6310
Periostin (POSTN)	**0.0037**	**0.0261**	**0.0125**	0.0838
Prostasin (PRSS8)	**0.0393**	0.0565	**0.0093**	**0.0087**
Tenascin-C (TNC)	**<0.0001**	**0.0004**	**0.0004**	**0.0018**
Tissue inhibitor of metalloproteinases 3 (TIMP-3)	0.0993	0.1311	**0.0445**	**0.0311**
Vascular cell adhesion molecule-1 (VCAM-1)	**0.0015**	**0.0107**	**0.0038**	**0.0175**

[a] $n = 40$ individuals with FOP and 40 unaffected individuals (controls); bolded p values are significant to <0.05; The gray shading fills the cells containing significant p-values and links these cells across the various statistical methods (e.g., shaded cells across an entire row indicates that a specific biomarker was statistically significant for all methods used).

Five analytes showed any statistical relationship with respect to disease activity. After adjustment for age group, only kallikrein-7 (KLK-7) showed significance across all four analyses. Fig. 3 shows the distribution of KLK-7 plasma levels between active and inactive FOP disease state, which is further stratified by individual donor levels (Supplemental Fig. S1). Using HCA (Fig. 2), analytes most similar to the flare-up status variable were leptin (LEP) and suppression of tumorigenicity 2 (ST2).

Network relationships among biomarkers

Network relationships among proteins significantly associated with FOP genotype and flare-up status were generated using IBM-Watson for Drug Discovery (IBM-WDD) (Fig. 4). The following proteins were used to generate relationship networks: ADIPOQ, serpin family A member 1 (SERPINA1), ferritin, IGFBP2, IGFBP7, KLK7, MMP3, TNC, VCAM1, MB, tumor necrosis factor (TNF), POSTN, LEP, ST2, and TNF receptor superfamily member 1A (TNFRSF1A). Not all proteins, however, could be placed in the networks based on our stringency criteria, including SERPINA1, ferritin, IGFBP2, IGFBP7, KLK7, TNC, MB, POSTN, and ST2. TNFRSF1A mapped to TNF and is represented by TNF in the network analysis (Fig. 4).

Discussion

Using a carefully collected and curated set of plasma samples from 40 FOP patients with the classic ACVR1^{R206H} mutation and 40 age- and sex-matched unaffected individuals, we identified several disease/genotype-related and flare-up-associated biomarkers of FOP that reflect a chronic pro-inflammatory state as well as an acute flare-up-related inflammatory phase of disease activity. We found that adiponectin (implicated in hypoxia, inflammation, and heterotopic ossification) and tenascin-C (an endogenous activator of innate immune signaling through the TLR4 pathway and a substrate for kallikrein-7) were highly correlated with FOP genotype, while kallikrein-7 was correlated with acute flare-up status.

Adiponectin was the biomarker most significantly associated with FOP genotype. Adiponectin is an adipocyte-specific hormone that has roles in the regulation of bone metabolism, is associated with ectopic osteogenesis, and is implicated in the metabolism of connective tissue progenitor cells.[15-18] Hypoxic and inflammatory adipocytes are involved in early HO formation,[19] and adiponectin levels are responsive to hypoxia and inflammation in human adipocytes.[20] Thus, it is tantalizing to speculate that adiponectin levels are indicative of microenvironmental priming and susceptibility to soft tissue injury in FOP and perhaps even early (ie, preclinical) lesions. IBM-WDD network analysis also suggests that adiponectin plays a role in the regulation of lesion formation.

Intriguingly, tenascin-C was also highly associated with FOP genotype. Tenascin-C is a large, extracellular matrix glycoprotein not expressed in most healthy adult tissues but rapidly and transiently induced at sites of tissue injury and inflammation.[21,22] Tenascin-C is implicated in bone morphogenesis and is regulated by the transforming growth factor-β.[23-25] Both BMP and Wnt signaling can induce tenascin-C expression, and the induction is mediated by MAP kinases.[26]

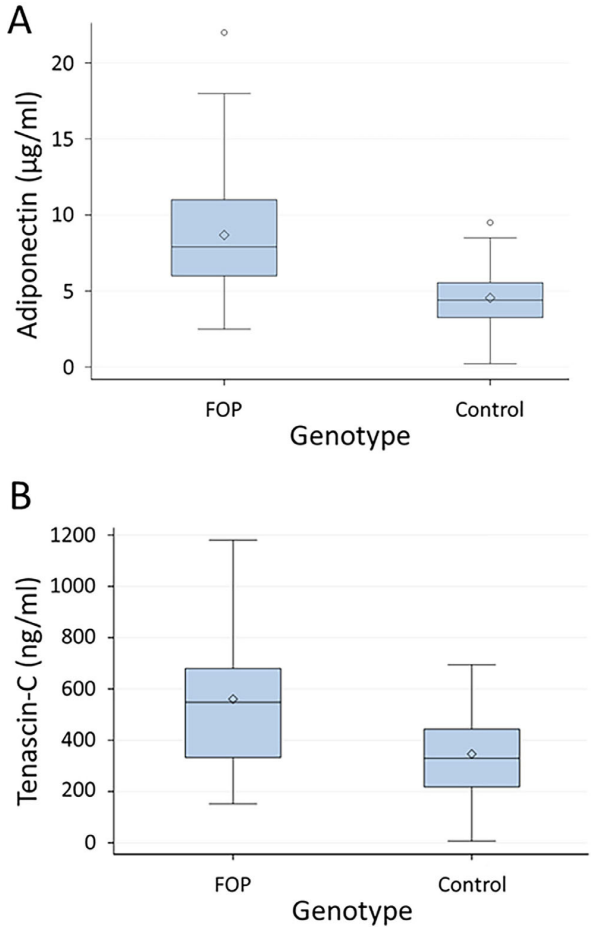

Fig. 1. (*A*) Boxplot of adiponectin levels in patients with fibrodysplasia ossificans progressiva (FOP; $n = 40$) and in unaffected controls ($n = 40$). (*B*) Boxplot of tenascin-C levels in patients with FOP ($n = 40$) and in unaffected controls ($n = 40$). Group means are denoted by diamond markers and outliers are denoted by circles. $p < 0.0001$.

Persistent tenascin-C expression is associated with pathologies such as chronic, non-healing wounds, autoimmune diseases, cancer, arthritis, and fibrotic diseases.[22,27] Tenascin-C induces synthesis of proinflammatory cytokines via NF-κB through endogenous activation of Toll-like receptor 4 (TLR4) and is a potent and endogenous activator of innate immunity and chronic inflammation.[21,28,29]

FOP patients have spontaneous flare-ups, flare-ups with exquisite sensitivity to trauma, as well as chronic disease exacerbation in the absence of flare-ups,[3] suggesting that dysregulated BMP pathway activation in FOP may cause altered immune responses.[10] Barruet and colleagues showed that dysregulated ACVR1 activity in FOP causes a chronic proinflammatory state mediated in part through the TLR4 pathway.[5] The finding that tenascin-C, an endogenous damage-associated molecular pattern (DAMP) that stimulates the TLR4 pathway, is elevated chronically in individuals with FOP strongly suggests that chronic inflammation mediated by the TLR4 pathway is a pathophysiologic signature of FOP. The exact DAMP(s) involved in the chronic pro-inflammatory signature of FOP have been elusive, but the findings described here may provide an important clue that can be explored in future studies.

Recent clinical and laboratory observations strongly suggest that the innate immune system induces flare-ups in the setting of dysregulated BMP signaling in FOP. Wang and colleagues found that inflammatory stimuli broadly activate TLR expression in FOP connective tissue progenitor cells (CTPCs) and that TLR4 signaling amplifies BMP pathway signaling through both ligand-dependent and -independent mechanisms. Importantly, Evolutionarily Conserved Signaling Intermediate in the Toll Pathway (ECSIT) integrates TLR4 injury signaling with dysregulated ACVR1 signaling in FOP CTPCs, thus providing a critical connection into cell autonomous integration of injury signals from the innate immune system with dysregulated response signals from the BMP signaling pathway.[10]

Of the 113 biomarkers interrogated, only kallikrein-7 (KLK-7) was significantly associated with flare-up activity across all ANOVA and logistical regression models studied. What is particularly intriguing is that tenascin-C, a chronic driver of innate immunity, is an endogenous substrate for kalikrein-7.[30] The kallikrein-kinin system is activated in inflammation and regulates prostaglandin synthesis,[31] suggesting that it may be involved in the early inflammatory stages of FOP lesion formation. That KLK-7 is decreased in active disease states suggests that TLR4 pro-inflammatory activity may be hyperactivated in acute flare-up states that are superimposed on a chronic inflammatory background.

Although HCA is not as strong a method as logistical regression modeling, HCA predicted that TNF pathways and leptin were related to FOP genotype and flare-up status, respectively, and both were linked by IBM-WDD to other biomarkers that were significantly associated with FOP genotype and flare-up status. These findings are consistent with the chronic pro-inflammatory milieu of FOP as well as the inflammatory nature of lipid mobilization in acute flare-ups and lesion formation.[19] The IBM-WDD network analysis suggests that adiponectin and leptin regulate inflammatory pathways[32-34] that downregulate TNFα expression, which in turn decreases VCAM-1 and MMP3 expression.[35,36] IL-6 is produced by both contracting skeletal muscle and adipose tissue and regulates adipogenesis and production of adiponectin.[37]

Barruet and colleagues reported that quiescent FOP subjects without clinically evident flare-ups showed increased serum levels of proinflammatory cytokines, including IL-3, IL-7, IL-8, and the anti-inflammatory cytokine IL-10.[5] These cytokines were not associated with FOP genotype or flare-up status in our study. Possible reasons for these inconsistent results include smaller sample size and no consideration for donor age in the Barruet and colleagues study or differing methods for biomarker quantification.

Taken together, our findings and those of others suggest a hypothetical schema that broadly integrates the identified biomarkers from this study with both the basal (chronic) and acute inflammatory stages of FOP and provides a preliminary map for navigating the complex pathophysiology of FOP (Fig. 5).

The current study is the first comprehensive evaluation of plasma biomarkers in classically affected FOP patients using carefully curated samples with respect to clinical flare-up status. However, we are keenly aware that there are limitations to this study. First, the selection of potential biomarkers was limited by currently available panels that were enriched for markers that might be dysregulated in FOP. Second, local (focal) effects may

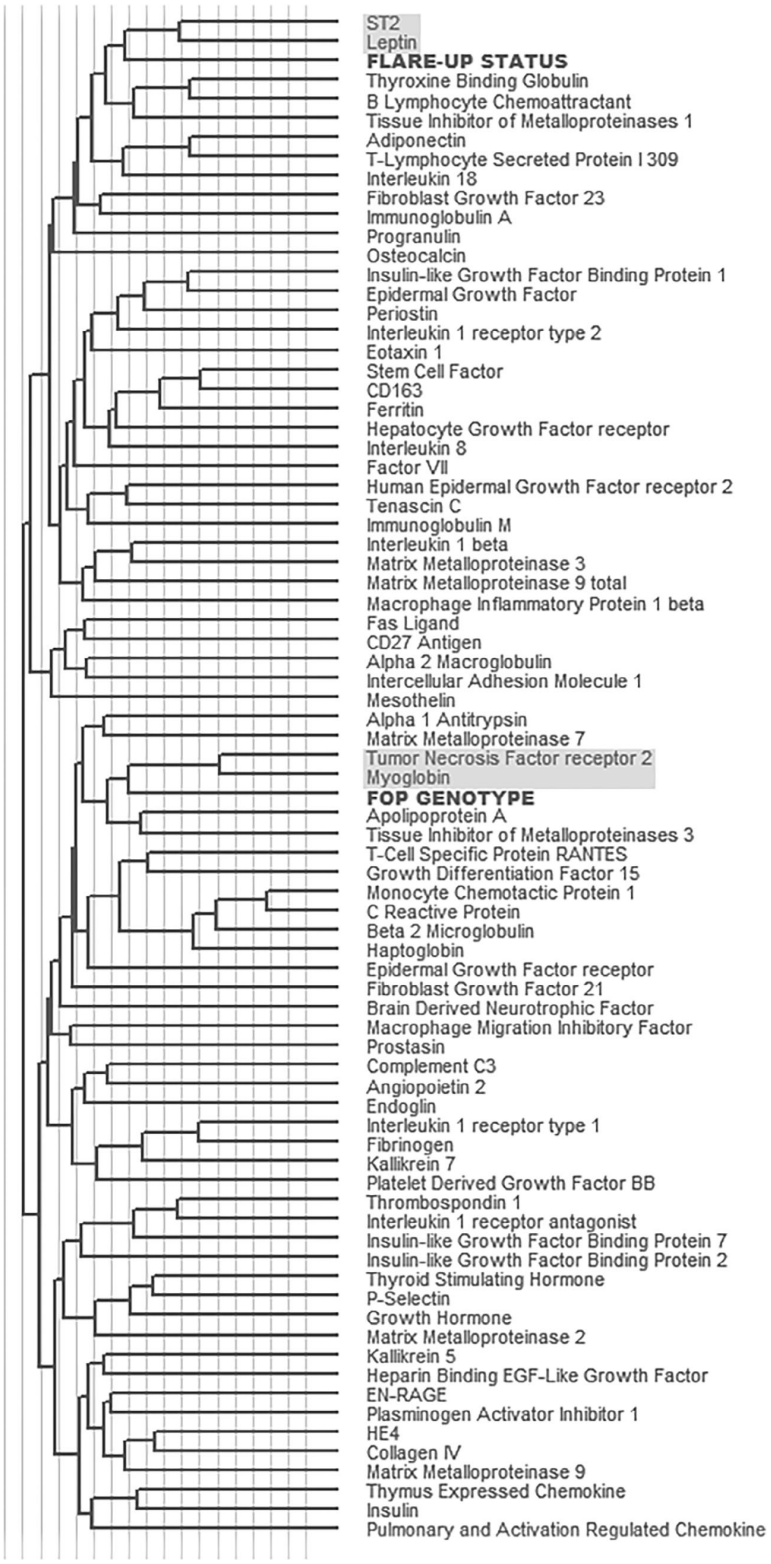

Fig. 2. Dendrogram from hierarchical clustering of analytes, fibrodysplasia ossificans progressiva (FOP) genotype, and flare-up status. Variables that share a common link near the ''branches'' of the dendrogram (right side) were merged earlier and are considered more closely related to those that are linked near the ''root'' of the dendrogram (left side). Biomarkers that are most closely related to flare-up status or FOP genotype are indicated by the shaded boxes.

Table 3. Biomarkers Associated With Fibrodysplasia Ossificans Progressiva (FOP) Disease Progression (ie, Flare-Ups)

	Model (FOP[a] active versus no active disease, $n = 40$)			
Biomarker	ANOVA (p value)	ANOVA [age group adjusted] (p value)	Logistical regression (p value)	Logistical regression [age group adjusted] (p value)
Beta-2-microglobulin (β2M)	0.0869	**0.0278**	0.0966	**0.0361**
Ferritin (FRTN)	**0.0490**	0.1440	0.0691	0.1491
Kallikrein-7 (KLK-7)	**0.0020**	**0.0006**	**0.0078**	**0.0064**
Periostin (POSTN)	**0.0041**	**0.0102**	**0.0262**	0.0556
Prostasin (PRSS8)	**0.0445**	0.0635	0.0592	0.0802

[a]$n = 20$ individuals with active FOP and 20 individuals with no active disease; bolded p values are significant to <0.05; The gray shading fills the cells containing significant p-values and links these cells across the various statistical methods (e.g., shaded cells across an entire row indicates that a specific biomarker was statistically significant for all methods used).

not be reflected in systemic, soluble biomarkers because of the volume of the lesion relative to circulatory (systemic) volume. Third, unlike in murine FOP studies in which the exact timing and location of lesion formation is controlled and predictable, the appearance and duration of flare-ups in humans with FOP is often spontaneous and unpredictable in duration, location, and evolution. Moreover, multiple lesions in various stages of evolution often exist simultaneously in humans[38] and thus might confound stage-specific biomarker identity.

Fourth, reports of biomarkers associated with early lesional events from genetic mouse models of FOP that display spontaneous HO are currently lacking. By limiting this analysis to studies in humans that include both spontaneous as well as trauma-induced flare-ups, we are more assured that the flare-up-associated trauma markers are real. In fact, a number of the analytes found in our study, such as alpha-1 antitrypsin, ferritin and osteocalcin, are classic acute-phase reactants, supporting the finding that at least a subset of the markers identified represent a non-specific inflammatory response found in both trauma-induced and spontaneous FOP lesions in humans. Fifth, although the prognostic importance of these findings is limited because of the absence of information on the clinical course of these patients subsequent to specimen acquisition, the stage-specific snapshot of plasma analytes is invaluable in understanding both the non-flare-up-related chronic pro-inflammatory stage as well as the clinically active, acute flare-up stage of FOP.

Sixth, these results are limited by the cross-sectional design of the study. Future analyses of promising biomarkers must be performed in fully curated plasma samples collected longitudinally. At this time, it is not possible to associate our biomarker results with clinical stage or volume of heterotopic ossification (HO). The clinical staging system we previously described[39] is still not validated, but it can potentially be applied to subjects with longitudinal data describing their disease progression, perhaps using information that has been collected in a global FOP natural history study (www.clinicaltrials.gov; Identifier NCT023322255). Also, the cohort used in our biomarkers study is a clinical cohort, and we do not routinely obtain imaging studies of high enough resolution or with broad enough anatomical coverage (ie, whole-body CT), and therefore we do not have volumetric HO data for the same patients analyzed in the current study. Correlating biomarker data with clinical staging or volumetric HO may also be feasible if current and future clinical trials adopt some of the more promising biomarkers identified here.

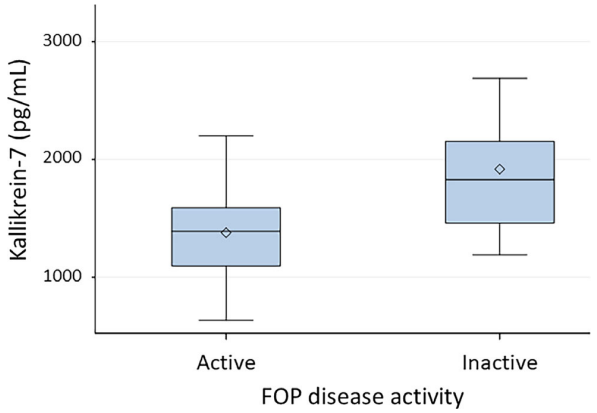

Fig. 3. Boxplot of kallikrein-7 levels in fibrodysplasia ossificans progressiva (FOP) patients with active disease ($n = 20$) and in those with inactive disease (ie, no flare-up symptoms for at least 1 year; $n = 20$). Group means are denoted by diamond markers and outliers are denoted by circles. $p = 0.0020$.

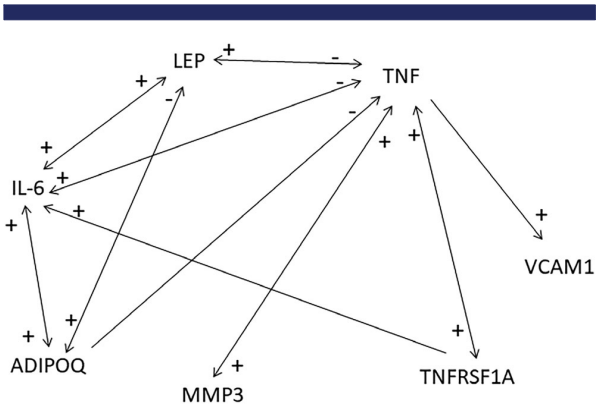

Fig. 4. IBM-WDD network analysis inputting proteins significantly associated with fibrodysplasia ossificans progressiva (FOP) genotype and flare-up status. Arrows indicate direction of influence. +, positive regulation; −, negative regulation.

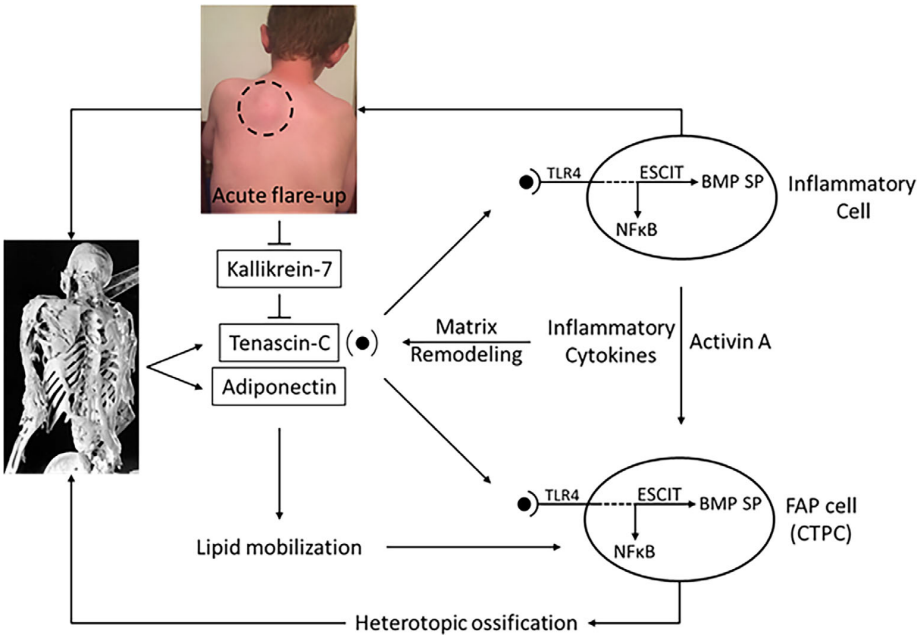

Fig. 5. Hypothetical schema integrates biomarker identification with the pro-inflammatory pathophysiology of fibrodysplasia ossificans progressiva (FOP). Arrows indicate positive influence; blunt-ended symbols indicate negative influence. Boxed entries indicate identified biomarkers. Filled circles indicate tenascin-C that stimulates extracellular toll-like receptor-4 (TLR-4; cupped symbol). ECSIT = evolutionarily conserved signal intermediate in the toll pathway. NFκB = nuclear factor-kappa B. BMP SP = bone morphogenetic protein signaling pathway. FAP cell = fibroadipogenic cell, a connective tissue progenitor cell (CTPC). FOP skeleton represents non-flare-up (basal) FOP. Picture showing acute inflammatory lesion of the back (dashed circle) represents an active flare-up.

Seventh and finally, biomarkers identified by our analysis span a wide variety of functions, including regulation of inflammation, extracellular matrix remodeling, angiogenesis, cellular adhesion and migration, and cellular metabolism. Given the known pleiotropic functions of the genes that encode these identified markers, it is also possible that their variation with genotype or disease state is related to as yet undiscovered roles for these proteins. Despite these limitations, this study identifies potential biomarkers that, with further verification, may enable the stratification of FOP stages by a minimally invasive blood draw, which poses little to no risk to individuals with FOP, and provides a real-world snapshot of both the chronic disease-related and the acute flare-up-related inflammatory nature of this complex disorder.

Disclosures

Alexion Pharmaceuticals, Inc., provided scientific review; however, the authors retain control and final authority of publication content and decisions. SB and JH were employees and shareholders of Alexion Pharmaceuticals, Inc., at the time the study was performed. SQ, KH, and HZ were employees of Blueprint Medicines at the time the study was performed. RJP and FSK are research investigators for Clementia Pharmaceuticals, Inc. (an Ipsen Company) and Regeneron Pharmaceuticals. RJP and FSK are the current and past presidents, respectively, of the International Clinical Council on FOP but have received no compensation in this role.

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Authors' roles: RJP, SB, and FSK conceived and designed the study. RJP wrote the initial manuscript with contributions from FSK and SB. RMW performed sample preparation and data collection. HW, SQ, KH, HZ, and RMW performed statistical and other analyses. RJP, FSK, HW, KE, and HZ interpreted the data. All authors contributed to the review of the manuscript and revisions. All authors approved the manuscript.

Author Contributions

Ruth McCarrick-Walmsley: Data curation; Formal analysis; Investigation; Writing-review & editing. **Shirley Qiu:** Formal analysis; Writing-review & editing. **Jeffrey Hunter:** Conceptualization; Funding acquisition; Investigation; Resources; Writing-review & editing. **Sharon Barr:** Conceptualization; Funding acquisition; investigation; Resources; Writing-review & editing. **Kevin He:** Formal analysis; Methodology; Writing-review &

editing. **Hui Zhang:** Formal analysis; Methodology; Writing-review & editing. **Robert Pignolo:** Conceptualization; Investigation; Data curation; Funding acquisition; Resources; Writing-review & editing. **Haitao Wang:** Formal analysis; Investigation; Methodology. **Frederick Kaplan:** Conceptualization; Investigation; Data curation; Funding acquisition; Resources; Writing-review & editing.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Pignolo RJ, Shore EM, Kaplan FS. Fibrodysplasia ossificans progressiva: clinical and genetic aspects. Orphanet J Rare Dis. 2011;6:80.
2. Pignolo RJ, Shore EM, Kaplan FS. Fibrodysplasia ossificans progressiva: diagnosis, management, and therapeutic horizons. Pediatr Endocrinol Rev. 2013;10(Suppl 2):437-448.
3. Pignolo RJ, Bedford-Gay C, Liljesthrom M, et al. The natural history of flare-ups in fibrodysplasia ossificans progressiva (FOP): a comprehensive global assessment. J Bone Miner Res. 2016;31(3):650-656.
4. Shore EM, Xu M, Feldman GJ, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet. 2006;38(5):525-527.
5. Barruet E, Morales BM, Cain CJ, et al. NF-kappaB/MAPK activation underlies ACVR1-mediated inflammation in human heterotopic ossification. JCI Insight. 2018;3(22):e122958.
6. Chakkalakal SA, Zhang D, Culbert AL, et al. An Acvr1 R206H knock-in mouse has fibrodysplasia ossificans progressiva. J Bone Miner Res. 2012;27(8):1746-1756.
7. Convente MR, Chakkalakal SA, Yang E, et al. Depletion of mast cells and macrophages impairs heterotopic ossification in an Acvr1 (R206H) mouse model of fibrodysplasia ossificans progressiva. J Bone Miner Res. 2018;33(2):269-282.
8. Kaplan FS, Glaser DL, Shore EM, et al. Hematopoietic stem-cell contribution to ectopic skeletogenesis. J Bone Joint Surg Am. 2007;89(2):347-357.
9. Upadhyay J, Xie L, Huang L, et al. The expansion of heterotopic bone in fibrodysplasia ossificans progressiva is activin A-dependent. J Bone Miner Res. 2017;32(12):2489-2499.
10. Wang H, Behrens EM, Pignolo RJ, Kaplan FS. ECSIT links TLR and BMP signaling in FOP connective tissue progenitor cells. Bone. 2018;109:201-209.
11. Kaplan F, Sawyer J, Connors S, et al. Urinary basic fibroblast growth factor. A biochemical marker for preosseous fibroproliferative lesions in patients with fibrodysplasia ossificans progressiva. Clin Orthop Relat Res. 1998;346:59-65.
12. Lindborg CM, Brennan TA, Wang H, Kaplan FS, Pignolo RJ. Cartilage-derived retinoic acid-sensitive protein (CD-RAP): a stage-specific biomarker of heterotopic endochondral ossification (HEO) in fibrodysplasia ossificans progressiva (FOP). Bone. 2018;109:153-157.
13. Hatz S, Spangler S, Bender A, et al. Identification of pharmacodynamic biomarker hypotheses through literature analysis with IBM Watson. PLoS One. 2019;14(4):e0214619.
14. Sanders JO, Qiu X, Lu X, et al. The uniform pattern of growth and skeletal maturation during the human adolescent growth spurt. Sci Rep. 2017;7(1):16705.
15. Shinoda Y, Yamaguchi M, Ogata N, et al. Regulation of bone formation by adiponectin through autocrine/paracrine and endocrine pathways. J Cell Biochem. 2006;99(1):196-208.
16. Mader R, Novofastovski I, Schwartz N, Rosner E. Serum adiponectin levels in patients with diffuse idiopathic skeletal hyperostosis (DISH). Clin Rheumatol. 2018;37(10):2839-2845.
17. Lees-Shepard JB, Yamamoto M, Biswas AA, et al. Activin-dependent signaling in fibro/adipogenic progenitors causes fibrodysplasia ossificans progressiva. Nat Commun. 2018;9(1):471.
18. Wosczyna MN, Biswas AA, Cogswell CA, Goldhamer DJ. Multipotent progenitors resident in the skeletal muscle interstitium exhibit robust BMP-dependent osteogenic activity and mediate heterotopic ossification. J Bone Miner Res. 2012;27(5):1004-1017.
19. Olmsted-Davis E, Gannon FH, Ozen M, et al. Hypoxic adipocytes pattern early heterotopic bone formation. Am J Pathol. 2007;170(2):620-632.
20. Wang B, Wood IS, Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. Pflugers Arch. 2007;455(3):479-492.
21. Marzeda AM, Midwood KS. Internal affairs: tenascin-C as a clinically relevant, endogenous driver of innate immunity. J Histochem Cytochem. 2018;66(4):289-304.
22. Udalova IA, Ruhmann M, Thomson SJ, Midwood KS. Expression and immune function of tenascin-C. Crit Rev Immunol. 2011;31(2):115-145.
23. Mackie EJ, Abraham LA, Taylor SL, Tucker RP, Murphy LI. Regulation of tenascin-C expression in bone cells by transforming growth factor-beta. Bone. 1998;22(4):301-307.
24. Mackie EJ, Tucker RP. Tenascin in bone morphogenesis: expression by osteoblasts and cell type-specific expression of splice variants. J Cell Sci. 1992;103(Pt 3):765-771.
25. Mackie EJ, Thesleff I, Chiquet-Ehrismann R. Tenascin is associated with chondrogenic and osteogenic differentiation in vivo and promotes chondrogenesis in vitro. J Cell Biol. 1987;105(6 Pt 1):2569-2579.
26. Morgan JM, Wong A, Yellowley CE, Genetos DC. Regulation of tenascin expression in bone. J Cell Biochem. 2011;112(11):3354-3363.
27. Gupta L, Bhattacharya S, Aggarwal A. Tenascin-C, a biomarker of disease activity in early ankylosing spondylitis. Clin Rheumatol. 2018;37(5):1401-1405.
28. Midwood K, Sacre S, Piccinini AM, et al. Tenascin-C is an endogenous activator of toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nat Med. 2009;15(7):774-780.
29. Zuliani-Alvarez L, Marzeda AM, Deligne C, et al. Mapping tenascin-C interaction with toll-like receptor 4 reveals a new subset of endogenous inflammatory triggers. Nat Commun. 2017;8(1):1595.
30. Yu Y, Prassas I, Dimitromanolakis A, Diamandis EP. Novel biological substrates of human kallikrein 7 identified through degradomics. J Biol Chem. 2015;290(29):17762-17775.
31. Lerner UH. Regulation of bone metabolism by the kallikrein-kinin system, the coagulation cascade, and the acute-phase reactants. Oral Surg Oral Med Oral Pathol. 1994;78(4):481-493.
32. Yuan B, Huang L, Yan M, et al. Adiponectin downregulates TNF-alpha expression in degenerated intervertebral discs. Spine (Phila Pa 1976). 2018;43(7):E381-E389.
33. Lee SW, Rho JH, Lee SY, et al. Leptin protects rat articular chondrocytes from cytotoxicity induced by TNF-alpha in the presence of cyclohexamide. Osteoarthr Cartil. 2015;23(12):2269-2278.
34. Qian H, Hausman GJ, Compton MM, Azain MJ, Hartzell DL, Baile CA. Leptin regulation of peroxisome proliferator-activated receptor-gamma, tumor necrosis factor, and uncoupling protein-2 expression in adipose tissues. Biochem Biophys Res Commun. 1998;246(3):660-667.
35. Li H, Yoneda M, Takeyama M, et al. Effect of infliximab on tumor necrosis factor-alpha-induced alterations in retinal microvascular endothelial cells and retinal pigment epithelial cells. J Ocul Pharmacol Ther. 2010;26(6):549-556.
36. Okada N, Fukagawa K, Takano Y, et al. The implications of the upregulation of ICAM-1/VCAM-1 expression of corneal fibroblasts on the pathogenesis of allergic keratopathy. Invest Ophthalmol Vis Sci. 2005;46(12):4512-4518.
37. Park AJ, Battaglino RA, Nguyen NMH, Morse LR. Associations between lean mass and leptin in men with chronic spinal cord injury: results from the FRASCI-muscle study. PLoS One. 2018;13(6):e0198969.
38. Kaplan FS, Tabas JA, Gannon FH, Finkel G, Hahn GV, Zasloff MA. The histopathology of fibrodysplasia ossificans progressiva. An endochondral process. J Bone Joint Surg Am. 1993;75(2):220-230.
39. Pignolo RJ, Kaplan FS. Clinical staging of fibrodysplasia ossificans progressiva (FOP). Bone. 2018;109:111-114.