Baseline Levels of Circulating Inflammatory Biomarkers Stratify Patients with Vitiligo Who Significantly Repigment after Treatment with Ruxolitinib Cream

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Background: Efficacy of ruxolitinib cream, a topical Jak1/Jak2 inhibitor, was demonstrated in a phase 2 trial in patients with vitiligo. Objective: This study aimed to characterize circulating inflammatory biomarker profiles in patients who demonstrated ≥50% improvement in facial Vitiligo Area Scoring Index scores by week 24 (group 1) and those who did not (group 2). Design: This was a posthoc analysis of a multicenter, randomized, double-blind, vehicle-controlled, phase 2 study in which screening was conducted between June 7, 2017 and March 21, 2018. Population: Patients aged between 18 and 75 years with vitiligo, including depigmentation affecting ≥0.5% of body surface area on the face and ≥3% of body surface area on nonfacial areas, were eligible. Intervention: Patients applied 1.5% ruxolitinib cream to lesions once or twice daily for 52 weeks. Main outcomes and measures: Patients were grouped by achievement of ≥50% improvement in facial Vitiligo Area Scoring Index at week 24. Proteomic analysis was performed on baseline serum samples. Results: Mean ± standard error facial Vitiligo Area Scoring Index in group 1 (n = 30) versus group 2 (n = 27) improved by 79.9 ± 4.0% versus 1.1 ± 7.3% and 91.9 ± 1.5% versus 25.1 ± 13.4% at weeks 24 and 52, respectively. Broad proteomic analysis revealed 76 proteins (of 1,104 tested) that were differentially expressed between groups 1 and 2 at baseline ($P < 0.05$). Ten distinct proteins were upregulated in group 1; 64 were elevated in group 2. Conclusion: This analysis identified potential differences between patients who achieved ≥50% improvement in facial Vitiligo Area Scoring Index at 24 weeks and those who did not that require deeper scientific interrogation and may be important in stratifying therapeutic benefit for patients with vitiligo. Trial Registration: The original study was registered at ClinicalTrials.gov, NCT03099304.

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INTRODUCTION

Vitiligo is an autoimmune disease characterized by areas of depigmented skin that affects up to 2% of the global population and is associated with a significantly reduced QOL (Krüger and Schallreuter, 2012; Morrison et al., 2017; Patel et al., 2019; Sheth et al., 2013). Currently, there are no United States Food and Drug Administration–approved treatments for repigmentation of vitiligo lesions, and most treatment paradigms rely on the use of corticosteroids, calcineurin inhibitors, narrowband UV light, or combination therapy.

The skin of patients with vitiligo is characterized by depigmented patches that result from the infiltration of affected areas with activated antigen-specific CD8+ T cells that drive melanocyte death and disease pathogenesis (Rodrigues et al., 2017). Recruitment of autoreactive CD8+ T cells to melanocytes is mediated by IFN-γ through the IFN-γ–induced chemokines CXCL9 and (Boniface et al., 2018) CXCL10, a signaling pathway regulated by Jak1 and Jak2 (Harris et al., 2012; Rashighi et al., 2014; Rashighi and Harris, 2015).

A cream formulation of ruxolitinib, a potent selective Jak1/Jak2 inhibitor, has previously shown preliminary efficacy in open-label studies (Joshipura et al., 2018; Rothstein et al., 2017) and was recently evaluated for the treatment of vitiligo in a randomized, double-blind, vehicle-controlled phase 2 study (NCT03099304) (Rosmarin et al., 2020). In the phase 2 clinical trial, treatment with ruxolitinib cream demonstrated significant repigmentation in patients, with 45.5 and 57.6% of patients who applied 1.5% ruxolitinib cream twice daily (BID) achieving ≥50% improvement in 50% improvement in facial Vitiligo Area Scoring Index (F-VASI) at week 24 and week 52, respectively (Rosmarin et al., 2020). Additional

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Abbreviations: BID, twice daily; F-VASI, facial Vitiligo Area Scoring Index; QD, once daily; Th, T helper; T-VASI, total Vitiligo Area Scoring Index

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in-depth analysis of patient demographics and baseline characteristics revealed that ruxolitinib cream was effective across patient characteristics and showed repigmentation of all body areas in patients with vitiligo, including the hands and feet (Hamzavi et al., 2022).

This study further investigated and characterized patients with vitiligo from the phase 2 clinical trial who achieved F-VASI50 at week 24 (group 1) after treatment with ruxolitinib cream compared with those who did not (group 2). Previous studies have demonstrated key roles for IFN-γ as well as the T-cell–recruiting chemokines CXCL9 and CXCL10 in driving disease pathogenesis (Richmond et al., 2017). In this study, we present the broad proteomic approaches used to further characterize and classify patients with vitiligo who achieved F-VASI50 at week 24.

RESULTS

Changes in F-VASI and total Vitiligo Area Scoring Index

This analysis focused on the treatment arms that received 1.5% ruxolitinib cream once daily (QD) and BID from the phase 2 study to reflect the concentration of ruxolitinib cream currently under evaluation in two phase 3 clinical trials (NCT04057573 and NCT04052425) (Rosmarin et al., 2022). Given the differential responses after treatment with ruxolitinib cream in the previously reported phase 2 clinical trial in patients with vitiligo (Rosmarin et al., 2020), patients were separated into subgroups for further analysis. Patients were classified as group 1 if they achieved F-VASI50 or greater at week 24 (n = 30); all other patients were classified as group 2 (n = 27) (Table 1). Within each subgroup, the percentage change in F-VASI and total Vitiligo Area Scoring Index (T-VASI) from baseline was determined for each patient to calculate the average change in disease severity for each subgroup. Table 2 and Figure 1 summarize the percentage changes in F-VASI and T-VASI for each subgroup. The F-VASI (mean ± standard error) scores of patients in group 1 who applied 1.5% ruxolitinib cream BID improved by 76.9 ± 4.0% and 91.9 ± 1.5% at weeks 24 and 52, respectively. In contrast, F-VASI improved by 1.1 ± 7.3% and 25.1 ± 13.4% among patients in group 2 at weeks 24 and 52, respectively.

Table 1. Patient Demographics and Baseline Clinical Characteristics

	Achieved F-VASI50 at Week 24			Did Not Achieve F-VASI50 at Week 24		
Characteristics	1.5% Ruxolitinib Cream QD (n = 15)	1.5% Ruxolitinib Cream BID (n = 15)	Total (n = 30)	1.5% Ruxolitinib Cream QD (n = 11)	1.5% Ruxolitinib Cream BID (n = 16)	Total (n = 27)
Age, y, mean ± SD	45.6 ± 9.3	46.9 ± 10.1	46.2 ± 9.6	52.4 ± 12.3	50.9 ± 14.1	51.5 ± 13.2
Female, n (%)	10 (66.7)	9 (60.0)	19 (63.3)	6 (54.5)	5 (31.3)	11 (40.7)
Race, n (%)						
White	11 (73.3)	13 (86.7)	24 (80.0)	10 (90.9)	15 (93.8)	25 (92.6)
Other[1]	4 (26.7)	2 (13.3)	6 (20.0)	1 (9.1)	1 (6.3)	2 (7.4)
Fitzpatrick skin type, n (%)						
I–III	9 (60.0)	12 (80.0)	21 (70.0)	9 (81.8)	12 (75.0)	21 (77.8)
IV–VI	6 (40.0)	3 (20.0)	9 (30.0)	2 (18.2)	4 (25.0)	6 (22.2)
Nonsegmental vitiligo, n (%)	15 (100)	14 (93.3)	29 (96.7)	11 (100)	15 (93.8)	26 (96.3)
Baseline F-VASI, mean ± SD	1.45 ± 1.08	1.16 ± 0.50	1.31 ± 0.84	1.59 ± 0.98	1.34 ± 1.02	1.44 ± 0.99
Baseline T-VASI, mean ± SD	19.1 ± 16.6	22.8 ± 19.3	20.9 ± 17.8	16.8 ± 19.1	16.5 ± 13.0	16.6 ± 15.4
Facial BSA,[2] mean ± SD, %	1.69 ± 1.06	1.45 ± 0.72	1.57 ± 0.90	1.71 ± 0.96	1.62 ± 0.99	1.66 ± 0.96
Total BSA, mean ± SD, %	21.95 ± 15.44	22.76 ± 18.76	22.35 ± 16.89	28.53 ± 24.93	20.54 ± 16.26	23.80 ± 20.19
Duration of disease, y, mean ± SD	18.3 ± 10.8	19.5 ± 13.6	18.9 ± 12.1	19.4 ± 17.3	12.6 ± 10.6	15.4 ± 13.9
Diagnosed in childhood, n (%)	1 (6.7)	6 (40.0)	7 (23.3)	2 (18.2)	4 (25.0)	6 (22.2)
Disease stability,[3] n (%)						
Stable	9 (60.0)	6 (40.0)	15 (50.0)	3 (27.3)	5 (31.3)	8 (29.6)
Progressive	6 (40.0)	9 (60.0)	15 (50.0)	8 (72.7)	11 (68.8)	19 (70.4)
Other autoimmune disorders,[4] n (%)						
Thyroid disorders	5 (33.3)	2 (13.3)	7 (23.3)	4 (36.4)	6 (37.5)	10 (37.0)
Juvenile diabetes mellitus	0	1 (6.7)	1 (3.3)	0	0	0
Pernicious anemia	0	0	0	0	0	0
Other	11 (73.3)	12 (80.0)	23 (76.7)	7 (63.6)	11 (68.8)	18 (66.7)
Previous therapy,[5] n (%)						
Topical calcineurin inhibitors	3 (20.0)	6 (40.0)	9 (30.0)	7 (63.6)	8 (50.0)	15 (55.6)
Topical corticosteroids	10 (66.7)	7 (46.7)	17 (56.7)	3 (27.3)	7 (43.8)	10 (37.0)
Excimer laser therapy	2 (13.3)	1 (6.7)	3 (10.0)	3 (27.3)	2 (12.5)	5 (18.5)
PUVA photochemotherapy	2 (13.3)	0	2 (6.7)	2 (18.2)	3 (18.8)	5 (18.5)

Abbreviations: BSA, body surface area; F-VASI, facial Vitiligo Area Scoring Index; PUVA, psoralen UVA; T-VASI, total Vitiligo Area Scoring Index.
[1]Includes Asian, Black/African-American, and others.
[2]Percentage of total BSA.
[3]Determination of disease stability was based on investigator judgment.
[4]Patients could report multiple autoimmune disorders.
[5]Patients could have used multiple previous lines of therapy.

Table 2. Percentage Change in F-VASI and T-VASI from Baseline

Treatment Group	All Patients			Patients Who Achieved F-VASI50 at Week 24 (Group 1)			Patients Who Did Not Achieve F-VASI50 at Week 24 (Group 2)		
	Week 12	Week 24	Week 52	Week 12	Week 24	Week 52	Week 12	Week 24	Week 52
Percentage change from baseline in F-VASI[1]									
1.5% QD	−22.6 ± 7.5 (n = 26)	−41.0 ± 9.3 (n = 26)	−55.6 ± 9.3 (n = 21)	−36.1 ± 10.3 (n = 15)	−67.9 ± 5.1 (n = 15)	−79.7 ± 6.7 (n = 12)	−4.3 ± 8.5 (n = 11)	−4.3 ± 15.2 (n = 11)	−23.5 ± 14.2 (n = 9)
1.5% BID	−17.3 ± 7.7 (n = 31)	−37.8 ± 8.1 (n = 31)	−57.3 ± 9.3 (n = 29)	−45.3 ± 7.0 (n = 15)	−76.9 ± 4.0 (n = 15)	−91.9 ± 1.5 (n = 14)	9.0 ± 9.5 (n = 16)	−1.1 ± 7.3 (n = 16)	−25.1 ± 13.4 (n = 15)
Percentage change from baseline in T-VASI[1]									
1.5% QD	−14.5 ± 5.4 (n = 26)	−28.1 ± 6.4 (n = 26)	−41.9 ± 7.8 (n = 21)	−20.1 ± 5.4 (n = 15)	−40.6 ± 5.7 (n = 15)	−55.8 ± 6.4 (n = 12)	−7.0 ± 10.4 (n = 11)	−11.0 ± 11.5 (n = 11)	−23.3 ± 14.4 (n = 9)
1.5% BID	−11.0 ± 4.0 (n = 31)	−22.9 ± 4.5 (n = 31)	−41.7 ± 4.9 (n = 29)	−22.7 ± 5.8 (n = 15)	−37.9 ± 5.9 (n = 15)	−57.6 ± 5.6 (n = 14)	−0.0 ± 3.9 (n = 16)	−8.8 ± 4.7 (n = 16)	−26.8 ± 5.8 (n = 15)

Abbreviations: BID, twice daily; F-VASI, facial Vitiligo Area Scoring Index; F-VASI50, ≥50% improvement in F-VASI; QD, once daily; T-VASI, total Vitiligo Area Scoring Index.

[1]Data are presented as the mean percentage change ± standard error.

Similarly, T-VASI improved by 37.9 ± 5.9% and 57.6 ± 5.6% at weeks 24 and 52, respectively, in group 1 among patients who applied 1.5% ruxolitinib cream BID, whereas group 2 experienced 8.8 ± 4.7% and 26.8 ± 5.8% improvements at the same time points.

Baseline differences in circulating inflammation based on achievement of F-VASI50 at week 24

We further investigated the differences between groups 1 and 2 by measuring the levels of 1,104 proteins in circulation at baseline, before treatment with ruxolitinib cream. To increase statistical power and minimize false discoveries, we combined the 1.5% ruxolitinib cream QD and BID groups for this analysis. Broad proteomic analysis identified 76 proteins that were differentially expressed between the subgroups at baseline (Table 3 and Figure 2). Ten distinct proteins were upregulated in group 1 at baseline, including IL-5 (fold change = 1.79, $P = 0.012$), IL-20 (1.27, $P = 0.010$), and IL-13 (1.22, $P = 0.047$) (Table 3). Conversely, 64 distinct proteins were elevated in group 2 at baseline, including HCLS1

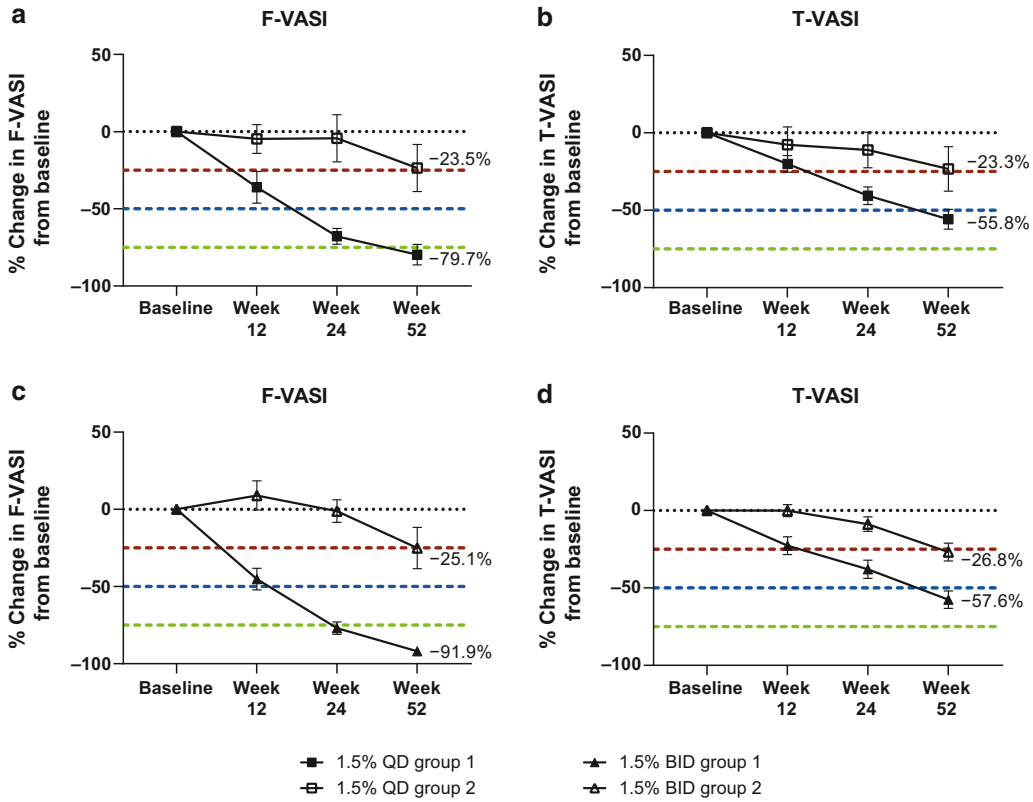

Figure 1. Differential efficacy of ruxolitinib cream between patients who did (group 1) and did not (group 2) achieve F-VASI50 at week 24. Average percentage change (±SEM) from baseline in (**a, c**) F-VASI and (**b, d**) T-VASI for each subgroup at weeks 12, 24, and 52. Red, blue, and green dotted lines represent 25, 50, and 75% reduction in score, respectively. BID, twice daily; F-VASI, facial Vitiligo Area Scoring Index; QD, once daily, T-VASI, total Vitiligo Area Scoring Index.

Table 3. Proteins that Were Increased at Baseline in Patients Who Achieved (Group 1) or Did Not Achieve (Group 2) F-VASI50 at Week 24

Protein	Protein Name	UNIPROT	Fold Change	Raw *P*-Value	FDR *P*-Value
Proteins upregulated in patients who achieved F-VASI50 at week 24 (group 1)					
IL-5	IL-5	Q01344	1.786	0.012	0.53
IL-5	IL-5	Q01344	1.754	0.014	0.53
CHRDL2	Chordin-like protein 2	Q6WN34	1.299	0.042	0.68
FCN2	Ficolin 2 precursor	Q15485	1.298	0.016	0.53
IL-20	IL-20	Q9NYY1	1.265	0.010	0.53
NID1	Nidogen 1	P14543	1.256	0.002	0.41
IL-13	IL-13	P35225	1.221	0.047	0.71
ARTN	Artemin	Q5T4W7	1.168	0.034	0.63
ACP6	Lysophosphatidic acid phosphatase type 6	Q9NPH0	1.166	0.038	0.64
TNXB	Tenascin-X	P22105	1.147	0.037	0.63
ITGB1BP1	Integrin beta-1-binding protein 1	O14713	1.05	0.050	0.72
Proteins upregulated in patients who did not achieve F-VASI50 at week 24 (group 2)					
HCLS1	Hematopoietic lineage cell-specific protein	P14317	1.701	0.002	0.41
AZU1	Azurocidin	P20160	1.592	0.009	0.53
PTN	Pleiotrophin	P63089	1.565	0.014	0.53
TANK	TRAF family member–associated NF-kappa-B activator	Q92844	1.558	0.012	0.53
CXCL9	C-X-C motif chemokine 9	Q07325	1.538	0.006	0.53
SRP14	Signal recognition particle 14 kDa protein	P37108	1.520	0.003	0.41
NMNAT1	Nicotinamide/nicotinic acid mononucleotide adenylyltransferase 1	Q9HAN9	1.479	0.045	0.71
CAPG	Macrophage-capping protein	P40121	1.460	0.005	0.53
PIK3AP1	Phosphoinositide-3-kinase adapter protein 1	Q9EQ32	1.449	0.010	0.53
TOP2B	DNA topoisomerase 2-beta	Q02880	1.445	0.023	0.61
NBN	Nibrin	O60934	1.443	0.035	0.63
EGLN1	Egl nine homolog 1	Q9GZT9	1.437	0.002	0.41
TRAF2	TNF receptor–associated factor 2	Q12933	1.429	0.003	0.41
CXCL10	C-X-C motif chemokine 10	P02778	1.403	0.030	0.61
PTX3	Pentraxin-related protein PTX3	P26022	1.401	0.020	0.60
NEMO	NF-kappa-B essential modulator	Q9Y6K9	1.393	0.030	0.61
NPM1	Nucleophosmin	P06748	1.387	0.003	0.41
DAPP1	Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide	Q9UN19	1.366	0.030	0.61
NADK	NAD kinase	O95544	1.359	0.036	0.63
BACH1	Transcription regulator protein BACH1	O14867	1.357	0.019	0.58
REN	Renin	P00797	1.357	0.011	0.53
DDX58	Antiviral innate immune response receptor RIG-I	O95786	1.333	0.010	0.53
IL-10	IL-10	P22301	1.332	0.013	0.53
CLEC4D	C-type lectin domain family 4 member D	Q8WXI8	1.332	0.015	0.53
VSIG2	V-set and immunoglobulin domain-containing protein 2	Q96IQ7	1.325	0.025	0.61
MB	Myoglobin	P02144	1.323	0.027	0.61
PRTN3	Myeloblastin	P24158	1.318	0.016	0.53
AARSD1	Alanyl-tRNA editing protein Aarsd1	Q9BTE6	1.305	0.038	0.64
ADM	Adrenomedullin	P35318	1.302	0.035	0.63
PXN	Paxillin	P35318	1.289	0.048	0.71
IL-10	IL-10	P22301	1.287	0.026	0.61
ILKAP	Integrin-linked kinase-associated serine/threonine phosphatase 2C	Q9H0C8	1.282	0.024	0.61
MSMB	Beta-microseminoprotein	P08118	1.271	0.029	0.61
LOX-1	Oxidized low-density lipoprotein receptor 1	P78380	1.269	0.028	0.61
APBB1IP	Amyloid beta A4 precursor protein-binding family B member 1-interacting protein	Q7Z5R6	1.267	0.024	0.61
IL-16	IL-16	Q14005	1.263	0.027	0.61
LAMP3	Lysosome-associated membrane glycoprotein 3	Q9UQV4	1.256	0.011	0.53
IL-32	IL-32	P24001	1.253	0.003	0.41
MAD1L1	Mitotic spindle assembly checkpoint protein MAD1	Q9Y6D9	1.248	0.001	0.41
IL-17RA	IL-17 receptor alpha	Q96F46	1.248	0.040	0.65
TREM1	Triggering receptor expressed on myeloid cells 1	Q9NP99	1.247	0.013	0.53
CRIP2	Cysteine-rich protein 2	P52943	1.241	0.015	0.53
LEPR	Leptin receptor	P48357	1.239	0.015	0.53

(*continued*)

Table 3. Continued

Protein	Protein Name	UNIPROT	Fold Change	Raw *P*-Value	FDR *P*-Value
NCR1	Natural cytotoxicity triggering receptor 1	O76036	1.235	0.003	0.41
TRIM21	E3 ubiquitin-protein ligase TRIM21	P19474	1.227	0.014	0.53
LAIR1	Leukocyte-associated immunoglobulin-like receptor 1	Q6GTX8	1.215	0.027	0.61
AGRP	Agouti-related protein	O00253	1.212	0.033	0.63
DFFA	DNA fragmentation factor subunit alpha	O00273	1.211	0.013	0.53
CLEC6A	C-type lectin domain family 6 member A	Q6EIG7	1.208	0.048	0.71
FCAR	Immunoglobulin alpha Fc receptor	P24071	1.205	0.012	0.53
ABHD14B	Protein ABHD14B	Q96IU4	1.205	0.020	0.60
FAM3B	Protein FAM3B	P58499	1.202	0.047	0.71
IFI30	Gamma-interferon–inducible lysosomal thiol reductase	P13284	1.199	0.015	0.53
CD302	CD302 antigen	Q8IX05	1.198	0.012	0.53
TF	Transferrin	Q06AH7	1.193	0.030	0.61
PHOSPHO1	Phosphoethanolamine/phosphocholine phosphatase	Q8TCT1	1.183	0.021	0.61
CD160	CD160 antigen	O88875	1.182	0.049	0.71
FLI1	Friend leukemia integration 1 transcription factor	Q01543	1.179	0.026	0.61
S100A11	Protein S100-A11	P31949	1.166	0.033	0.63
TGFR-2	TGF-β receptor type-2	P37173	1.163	0.036	0.63
PSME1	Proteasome activator complex subunit 1	Q06323	1.159	0.026	0.61
TRAIL-R2	TNF ligand superfamily member 10	P50591	1.151	0.033	0.63
TNF-R1	TNF receptor 1		1.149	0.024	0.61
BNP	Brain natriuretic peptide	P16860	1.121	0.047	0.71
FKBP4	Peptidyl-prolyl *cis-trans* isomerase FKBP4	Q02790	1.107	0.017	0.56

Abbreviations: FDR, false discovery rate; F-VASI50, ≥50% improvement in facial Vitiligo Area Scoring Index.

(fold change = 1.70, $P = 0.002$), CXCL9 (1.54, $P = 0.006$), PIK3AP1 (1.45, $P = 0.010$), CXCL10 (1.40, $P = 0.030$), and IL-16 (1.26, $P = 0.027$).

On the basis of the proteomic differences observed at baseline, we further evaluated the baseline expression of additional proteins previously shown to be associated with vitiligo pathogenesis and T-cell–mediated inflammation. Circulating levels of galectin-9, IL-12B, and IFN-γ were below the limit of detection (data not shown), and no significant differences were observed in the baseline levels of CXCL10, IL-2R, granzyme B, CCL18, and MIP3β between group 1 and group 2 (Table 4 and Figure 3a–e). In contrast, baseline levels of CXCL9 were significantly ($P = 0.016$) greater in group 2 (777.8 ± 149.4 pg/ml) than in group 1 (469.4 ± 53.1 pg/ml) (Figure 3f). Computationally combining the levels of various combinations of proteins found that the combination of CXCL9, CXCL10, and MIP3β was associated with significant ($P = 0.004$) baseline differences between subgroups (Figure 3g and h) that were capable of stratifying patients with vitiligo before treatment with ruxolitinib cream (area under the curve = 0.72, $P = 0.005$).

DISCUSSION

The efficacy of ruxolitinib cream in a randomized, double-blind, phase 2 study for adult patients with vitiligo in the United States was recently described (Rosmarin et al., 2020). Using the primary endpoint from the clinical trial (F-VASI50 at week 24), we stratified patients into two subgroups: those who achieved F-VASI50 at week 24 (group 1) and those who did not (group 2). Comparing the percentage change in F-VASI after treatment, we observed significantly greater reductions in group 1 than in group 2, with 91.9% improvement in F-VASI of group 1 at week 52 versus 25.1% in group 2. This corresponded to similar responses in T-VASI, where group 1 experienced a 57.6% reduction in T-VASI over 52 weeks compared with 26.8% in group 2. Given the differences in response between the subgroups after treatment with ruxolitinib cream, we further investigated whether differences at baseline (before treatment) could identify patients with a higher probability of response.

Vitiligo is an autoimmune disease characterized by a combination of systemic and local skin inflammation. Specifically, previous studies have highlighted the key role of Jak-mediated IFN-γ signaling in driving disease pathogenesis (Rashighi et al., 2014; Rashighi and Harris, 2015). We previously demonstrated that 52 weeks of treatment with ruxolitinib cream was associated with reduced circulating levels of IFN-γ–associated proteins CXCL10, CCL18, and CD27 (Rosmarin et al., 2020). Using broad proteomic analysis, we investigated 1,104 proteins in circulation and found that 10 distinct proteins were increased in group 1 at baseline, whereas 64 proteins were increased in group 2. Group 1 was characterized by increased levels of the T helper (Th) 2 cytokines IL-5 and IL-13 as well as IL-20, which are associated with keratinocyte proliferation and production of proinflammatory cytokines and chemokines in the skin (Rich and Kupper, 2001). Interestingly, group 2 was characterized by increased levels of IFN-γ–associated proteins CXCL9 and CXCL10 as well as other inflammatory cytokines such as IL-17RA, IL-16, and IL-32. IL-16, formerly known as lymphocyte chemoattractant factor, is a chemoattractant for CD4+ cells and has been shown to be elevated in the sera of

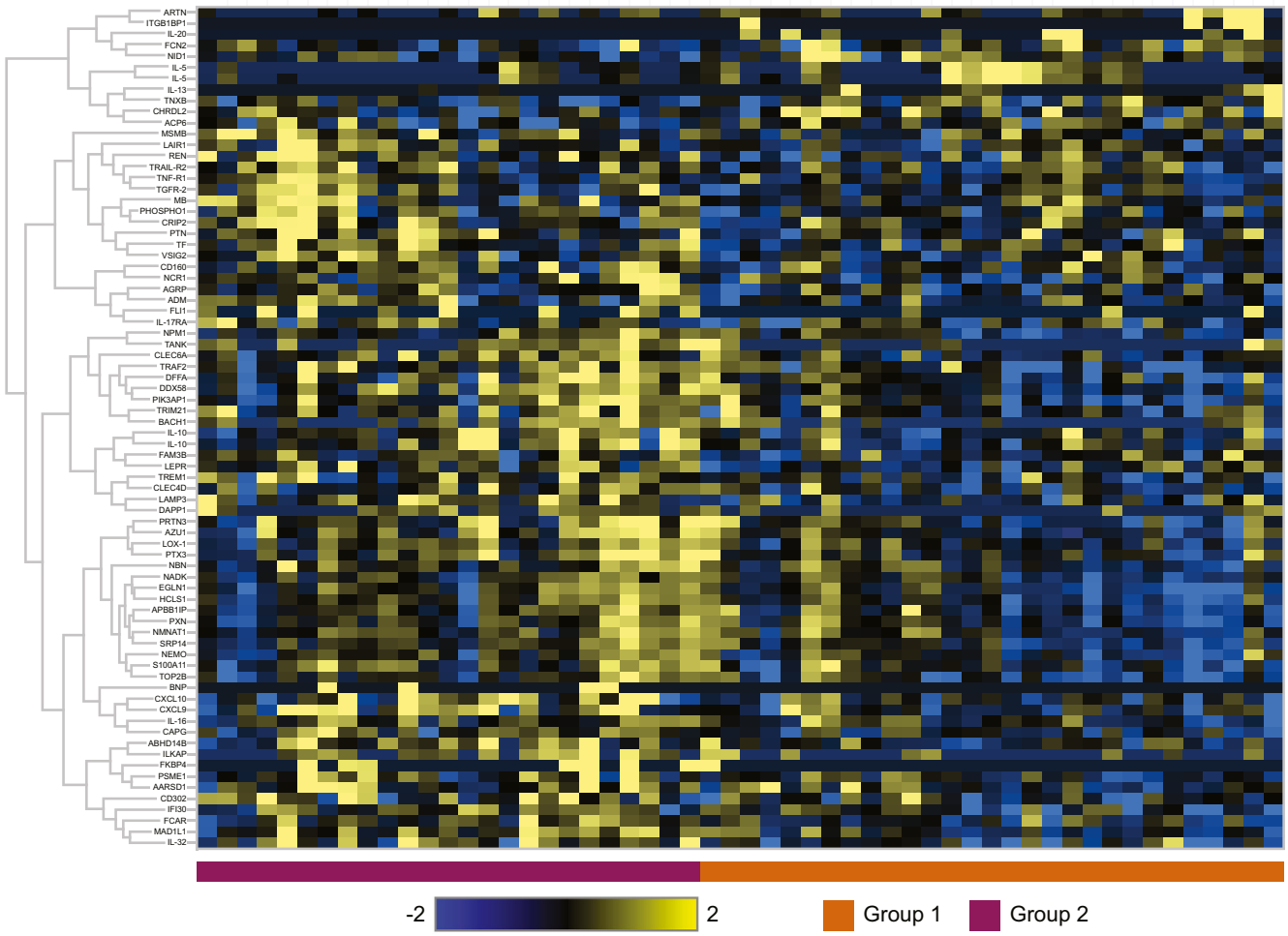

Figure 2. Heat map of proteins. Hierarchical clustering of the samples of the 76 proteins that were differentially expressed at baseline between patients who achieved F-VASI50 at week 24 (group 1, orange boxes) and patients who did not achieve F-VASI50 at week 24 (group 2, purple boxes). F-VASI50, ≥50% improvement in facial Vitiligo Area Scoring Index.

Table 4. Levels of Circulating Inflammatory Markers at Baseline

	CCL18	CXCL10	CXCL9	Granzyme B	IL-2R	MIP3β
Patients who achieved F-VASI50 at week 24 (group 1)[1]	42,802 ± 3746	165.9 ± 12.3	469.4 ± 53.1	7.3 ± 0.6	778.0 ± 103.3	169.5 ± 12.7
Patients who did not achieve F-VASI50 at week 24 (group 2)[1]	49,915 ± 5780	197.5 ± 17.2	777.8 ± 149.4	11.4 ± 4.1	892.0 ± 138.2	210.3 ± 21.5
P-value[2]	0.39	0.11	0.016	0.74	0.74	0.33
AUC	0.5688	0.6263	0.6878	0.5271	0.5265	0.5780

Abbreviations: AUC, area under the curve; F-VASI50, ≥50% improvement in facial Vitiligo Area Scoring Index.
[1]All values reported as mean ± standard error pg/ml.
[2]Statistical significance determined using *t*-test.

patients with systemic sclerosis (Kawabata et al., 2020), bullous pemphigoid (Frezzolini et al., 2004), and psoriasis (Purzycka-Bohdan et al., 2016). IL-32 is a proinflammatory cytokine that can be stimulated by IFN-γ, TNF-α, and Th1 cells but not Th2, regulatory T, or Th17 cells (Meyer et al., 2010). Increased expression of IL-32 has been reported in patients with alopecia areata (Fuentes-Duculan et al., 2016) and hidradenitis suppurativa (Thomi et al., 2017). Most recently, IL-32 was categorized in Th1/Th17 cluster and serves a role in maintaining a state of chronic inflammation in lesional hidradenitis suppurativa skin (Thomi et al., 2017). Finally, levels of the cytotoxic factor granzyme B did not differ between the two groups.

Our proteomic data suggest that patients in groups 1 and 2 have different circulating inflammatory phenotypes that may influence their response to ruxolitinib cream. Specifically, patients in group 1 have increased Th2-related inflammation, whereas group 2 is skewed toward Th1/Th17-mediated

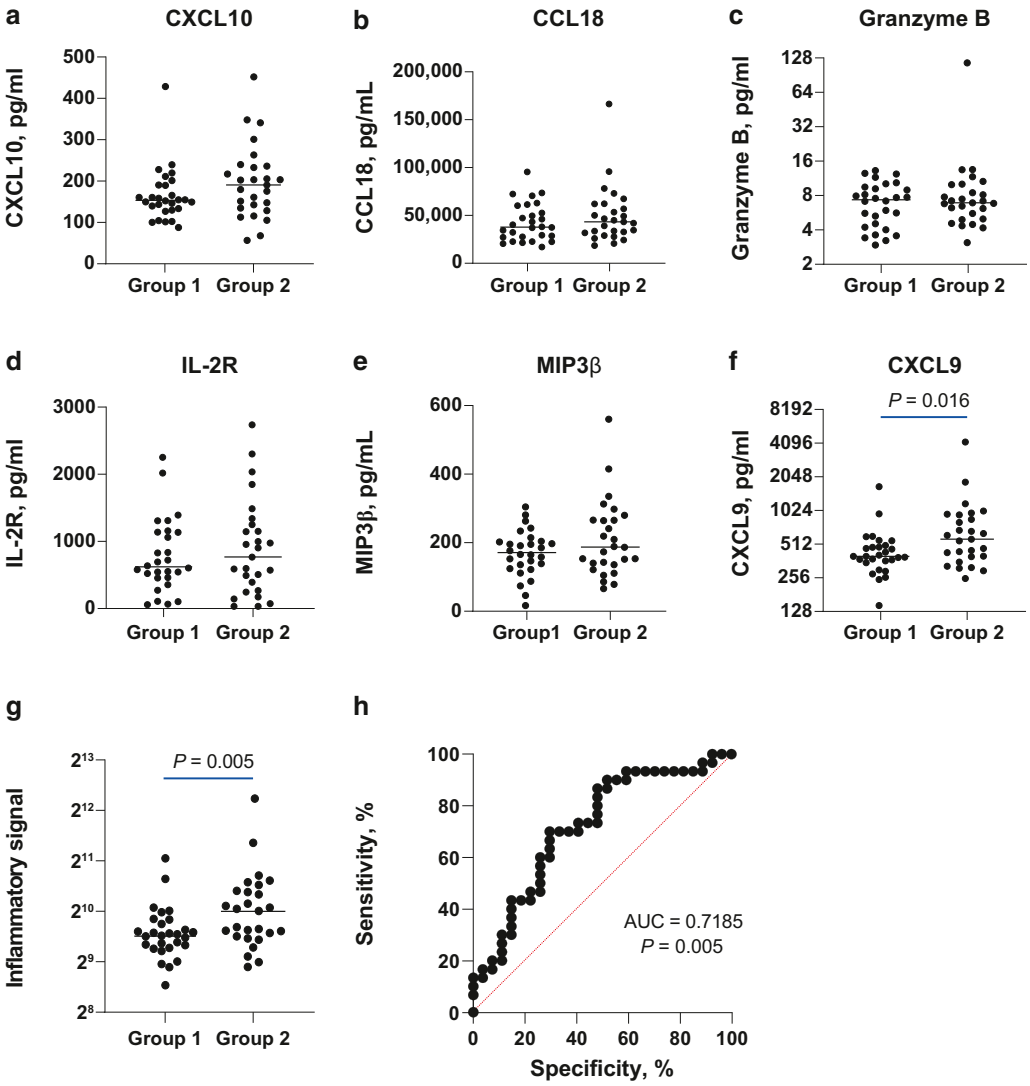

Figure 3. Stratification by baseline inflammatory signal. Baseline levels of (**a**) CXCL10, (**b**) CCL18, (**c**) granzyme B, (**d**) IL-2R, (**e**) MIP3b, and (**f**) CXCL9. CXCL10, CXCL9, and MIP3b were combined to generate an (**g**) inflammatory signal, and (**h**) the AUC was calculated between groups to determine the ability to accurately stratify participants on the basis of baseline inflammatory signals. Group 1: patients who achieved F-VASI50 at week 24; group 2: patients who did not achieve F-VASI50 at week 24. AUC, area under the curve; F-VASI50, ≥50% improvement in facial Vitiligo Area Scoring Index; IL-2R, IL-2 receptor.

inflammation. Elevated Th2 inflammatory signatures have previously been reported in serum samples from patients with vitiligo (Czarnowicki et al., 2019). Interestingly, several studies have revealed a significant relationship between atopic dermatitis, a prototypical Th2-driven disease, and vitiligo (Acharya and Mathur, 2020; Drucker et al., 2017; Lim et al., 2022; Mohan and Silverberg, 2015; Okano et al., 1986). In addition, patients with vitiligo often report pruritus (Silverberg and Silverberg, 2013). Ruxolitinib cream was recently shown to be highly efficacious in multiple clinical trials in patients with atopic dermatitis (Kim et al., 2020a, 2020b; Papp et al., 2021). Overlap in immunopathogenesis of the two diseases has been speculated, especially with regard to the heterogeneity of immune pathways in atopic dermatitis, including Th1 and Th17 pathways, although no common Jak pathway has been identified yet (Acharya and Mathur, 2020). In a recent study, T cells isolated from perilesional skin in patients with vitiligo and activated by an anti-CD3 antibody secreted Th1- (IFN-γ) and Th2- (mainly IL-13 and IL-5) related cytokines, inducing melanocytes to upregulate genes related to type 1 and type 2 immune responses (Martins et al., 2022). Furthermore, expression of IL-13 in serum samples from patients with vitiligo was negatively correlated with duration of disease, whereas expression of IFN-γ was positively correlated with disease duration, potentially rendering lesions in patients who had vitiligo for longer periods less responsive to immunomodulatory therapies (Czarnowicki et al., 2019).

Although vitiligo pathogenesis is primarily driven by IFN-γ, Jak-independent pathways have also been implicated. In addition, TNF-α, another Th1 cytokine, mediates apoptosis of melanocytes through the Jak-independent death domain pathway and induces the expression of intercellular adhesion molecule-1, which recruits T cells to melanocytes, and autophagy (Camara-Lemarroy and Salas-Alanis, 2013; Singh et al., 2021). Patients with either localized or generalized

Table 5. List of Institutional Review Boards and/or Independent Ethics Committees

IRB or IEC	Address	Chair	Study Site(s)
The Committee on the Protection of Human Subjects	Hoppin Street, Suite 1.300 Providence, RI 02903	IRB Chair not disclosed	Rhode Island Hospital, RI
Copernicus Group	5000 CentreGreen Way, Suite 200 Cary, NC 27513	Glenn Veit[1]	ActivMed Practices & Research, NH Arlington Research Center, TX Burke Pharmaceutical Research, AZ Central Sooner Research, OK Clinical Research Center of Connecticut, CT Dawes Fretzin Dermatology Group, IN The Dermatology Clinical Research Center of San Antonio, TX Dermatology Research Associates — Los Angeles, CA Dermatology Specialists, CA DS Research, IN Icahn School of Medicine at Mount Sinai Medical Center— Dermatology Associates, NY Leavitt Medical Associates of Florida, Ameriderm Research, FL Menter Dermatology Research Institute, TX Northwest Arkansas Clinical Trials Center, HullbDermatology and Aesthetics, AZ The Vitiligo & Pigmentation Institute of Southern California, CA
Henry Ford Hospital	2799 W Grand Boulevard Detroit, MI 48202	Kelly Jones	Henry Ford Hospital, MI
Tulane University Human Research Protection Office	1440 Canal Street, Suite 1705 New Orleans, LA 70112	IRB Chair not disclosed	Tulane University Health Sciences Center, LA
University of Massachusetts Medical School Committee for the Protection of Human Subjects in Research	362 Plantation Street, Seventh Floor Worcester, MA 01605	Carol Bova	University of Massachusetts Medical School, MA
University of Texas Southwestern Medical Center IRB	5323 Harry Hines Boulevard Dallas, TX 75390	Ahamed Idris	University of Texas Southwestern Medical Center, TX
Wake Forest University Health Sciences IRB	Medical Center Boulevard Winston Salem, NC 27157	Sally Bulla	Wake Forest University Health Sciences, NC
Washington University IRB	660 S Euclid, Campus Box 8089 St Louis, MO 63110	Jennifer Gartland	Washington University School of Medicine, MO
Western IRB	1019 39th Avenue SE, Suite 120 Puyallup, WA 98374	Glenn Veit[1]	University of Alabama at Birmingham, AL Emory University, Atlanta, GA Northwestern University, IL Tufts Medical Center, MA

Abbreviations: IEC, independent ethics committee; IRB, institutional review board.
[1]Study protocol was reviewed under the Western IRB-Copernicus Group Single Review Solution.

vitiligo were reported to have increased serum levels of TNF-α, especially in active vitiligo (Karagün and Baysak, 2020; Ranjkesh et al., 2021; Sushama et al., 2019). In a recent meta-analysis, the −308(G>A) polymorphism (rs1800629) in the *TNFA* promoter, which is associated with increased expression of TNF-α (Kroeger et al., 1997), was also associated with increased susceptibility to vitiligo overall and within Egyptian, Asian, and Middle Eastern populations and increased vitiligo activity in the North American population (Giri et al., 2022). In our study, we found that expression of TNF-α receptor 1 and TRAF2 were upregulated in group 2 compared with that in group 1, indicating a potential inverse relationship between baseline TNF-α levels and achieving clinical response. IL-17, a Th17 cytokine, mediates signaling through an NF-κB activator 1–dependent pathway that is independent of Jak signaling (Gaffen, 2009). Serum levels of IL-17 were elevated in patients with vitiligo compared with those in healthy controls (Karagün and Baysak, 2020; Tomaszewska et al., 2022) and were also positively correlated with disease duration, activity, and extent (Czarnowicki et al., 2019; Sushama et al., 2019). Importantly, IL-17RA was upregulated in group 2 relative to that in group 1, indicating that baseline serum IL-17 levels may also have an inverse relationship with achieving clinical response. Our results reflect heterogeneity in the presence of serum cytokines in patients with vitiligo that may predict differences in responsiveness to ruxolitinib cream, although it is unclear what role this heterogeneity plays in the pathogenesis of disease in the skin. As such, the correlation of serum cytokine levels in our study is not intended to identify drug targets for vitiligo treatment but rather provides an opportunity to further investigate accessible biomarkers that predict treatment response. Overall, this study identified potential differences between patients with vitiligo who achieved F-VASI50 after treatment with ruxolitinib cream for 24 weeks and patients who needed longer treatment time to achieve similar benefits. Proteomic differences at baseline suggest distinct inflammatory profiles; however, these observations were

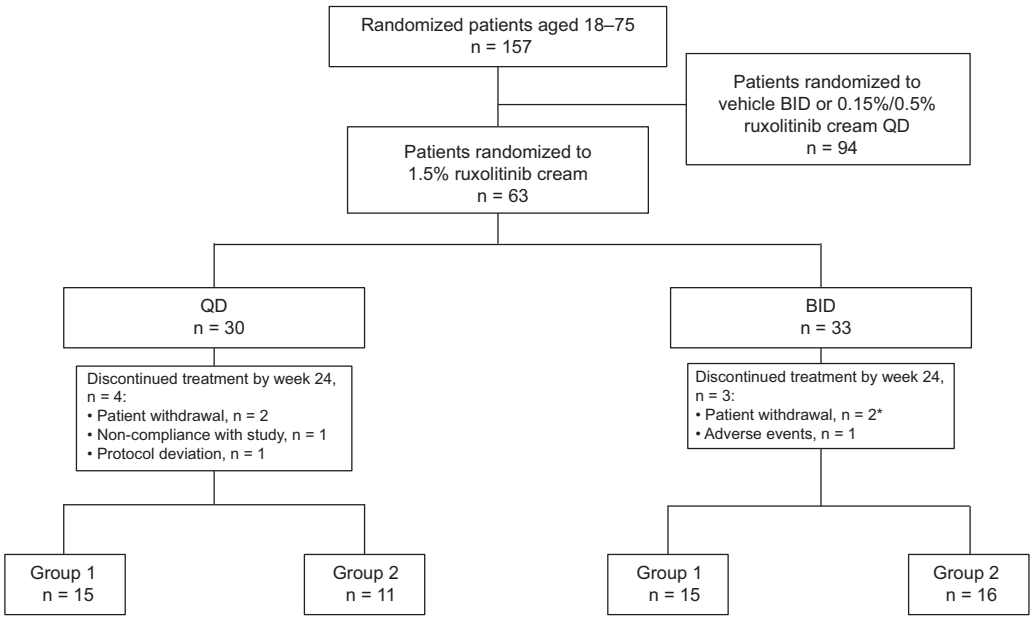

Figure 4. Patient disposition. Asterisk (*) denotes one patient who withdrew from treatment on day 162 and had a week 24 visit on record; the patient achieved F-VASI50 at week 24 and was included in group 1 for analysis of baseline biomarkers. BID, twice daily, F-VASI50, ≥50% improvement in facial Vitiligo Area Scoring Index; QD, daily.

evaluated in a limited number of patients within each subgroup and should be further investigated and validated in larger studies. Interestingly, these observations suggest that patients who achieved F-VASI50 at week 24 are characterized by less IFN-γ–mediated inflammation in circulation. Combined, these data support the observed therapeutic benefit of Jak1/Jak2 inhibition by ruxolitinib cream in patients with vitiligo and suggest that deeper interrogation of proteomic information could reveal biomarkers capable of stratifying patients at baseline into groups with different patterns of systemic inflammation. This additional information may be helpful in not only managing vitiligo directly but also the psychosocial impact of the disease on the individual during treatment.

MATERIALS AND METHODS

Patients and study design

This 52-week, randomized, double-blind, phase 2 study (NCT03099304) enrolled adult patients (aged 18–75 years) with vitiligo that included depigmentation of ≥0.5% of body surface area on the face and ≥3% of body surface area on nonfacial areas. Patients were screened for eligibility between June 7, 2017 and March 21, 2018. Key inclusion and exclusion criteria were described previously (Rosmarin et al., 2020).

Randomization and masking

Overall, 157 patients were equally randomized to receive ruxolitinib cream (1.5% BID, 1.5% QD, 0.5% QD, or 0.15% QD) or vehicle BID for 24 weeks. The randomization and double-blind masking processes were described previously (Rosmarin et al., 2020).

Procedure and follow-up

During the 24-week double-blind period, eligible patients applied either vehicle or ruxolitinib cream according to their assigned treatment group. After 24 weeks, patients either were rerandomized (patients who initially received vehicle and those treated with 0.15% ruxolitinib cream QD who did not achieve ≥25% improvement from baseline in F-VASI at week 24) to one of the three higher ruxolitinib cream doses or maintained their original dose until week 52. Additional details regarding the study design have been previously published (Rosmarin et al., 2020). Rerandomized patients were not included in this analysis. Patients were instructed to treat lesions constituting a maximum of 20% of baseline total body surface area for the duration of the study.

This study was conducted in accordance with the Declaration of Helsinki; written informed consent was obtained for all patients. The institutional review board of each site approved the study protocol (Table 5).

Patient flow

Patient flow during the double-blind period is presented in Figure 4.

Proteomic analysis

Proteomic analysis was performed on serum from patients who applied 1.5% ruxolitinib cream QD or BID. Samples were drawn at baseline. The proximity extension assay platform by Olink Proteomics consisting of 12 panels (oncology II, cardiovascular disease II, cardiovascular disease III, inflammation I, neurology I, immune response, metabolism, organ damage, cardiometabolic, cell regulation, development, and neuroexploratory) from the Olink Discovery platform (Watertown, MA) was used to conduct high-content (1,000 proteins) multiplex proteomic analysis in serum samples collected from all enrolled patients. In this assay, a pair of oligonucleotide-labeled antibodies, ProSeeK probes, are allowed to bind pairwise to the target protein present in the sample in a homogeneous assay. When the two ProSeeK probes are in close proximity, a new PCR-target sequence is formed by a proximity-dependent DNA polymerization event. The resulting sequence is subsequently detected and quantified using standard real-time PCR. Data are presented as normalized protein expression in $\log_2$ scale (Wik et al., 2021).

Table 6. GraphPad Prism Sensitivity and Specificity Calculation Table Used for ROC/AUC Determination

Score	Sensitivity, %	95% CI	Specificity, %	95% CI	Likelihood Ratio
<184.5	6.667	1.185–21.32%	100.0	87.54–100.0%	—
<420.8	10.00	3.460–25.62%	100.0	87.54–100.0%	—
<473.4	13.33	5.310–29.68%	100.0	87.54–100.0%	—
<484.1	13.33	5.310–29.68%	96.30	81.72–99.81%	3.600
<500.6	16.67	7.337–33.56%	96.30	81.72–99.81%	4.500
<509.4	16.67	7.337–33.56%	92.59	76.63–98.68%	2.250
<530.0	20.00	9.505–37.31%	92.59	76.63–98.68%	2.700
<569.4	20.00	9.505–37.31%	88.89	71.94–96.15%	1.800
<600.0	23.33	11.79–40.93%	88.89	71.94–96.15%	2.100
<613.5	26.67	14.18–44.45%	88.89	71.94–96.15%	2.400
<619.1	30.00	16.66–47.88%	88.89	71.94–96.15%	2.700
<631.1	30.00	16.66–47.88%	85.19	67.52–94.08%	2.025
<643.9	33.33	19.23–51.22%	85.19	67.52–94.08%	2.250
<652.3	36.67	21.87–54.49%	85.19	67.52–94.08%	2.475
<663.0	40.00	24.59–57.68%	85.19	67.52–94.08%	2.700
<679.6	43.33	27.38–60.80%	85.19	67.52–94.08%	2.925
<696.8	43.33	27.38–60.80%	81.48	63.30–91.82%	2.340
<706.5	43.33	27.38–60.80%	77.78	59.24–89.39%	1.950
<716.4	46.67	30.23–63.86%	77.78	59.24–89.39%	2.100
<722.8	46.67	30.23–63.86%	74.07	55.32–86.83%	1.800
<726.0	50.00	33.15–66.85%	74.07	55.32–86.83%	1.929
<736.8	53.33	36.14–69.77%	74.07	55.32–86.83%	2.057
<748.7	56.67	39.20–72.62%	74.07	55.32–86.83%	2.186
<754.8	60.00	42.32–75.41%	74.07	55.32–86.83%	2.314
<758.0	60.00	42.32–75.41%	70.37	51.52–84.15%	2.025
<761.2	63.33	45.51–78.13%	70.37	51.52–84.15%	2.138
<768.1	66.67	48.78–80.77%	70.37	51.52–84.15%	2.250
<774.8	70.00	52.12–83.34%	70.37	51.52–84.15%	2.363
<779.8	70.00	52.12–83.34%	66.67	47.82–81.36%	2.100
<785.6	70.00	52.12–83.34%	62.96	44.23–78.47%	1.890
<789.7	70.00	52.12–83.34%	59.26	40.73–75.49%	1.718
<795.3	73.33	55.55–85.82%	59.26	40.73–75.49%	1.800
<810.6	73.33	55.55–85.82%	55.56	37.31–72.41%	1.650
<839.4	73.33	55.55–85.82%	51.85	33.99–69.26%	1.523
<884.3	76.67	59.07–88.21%	51.85	33.99–69.26%	1.592
<915.1	80.00	62.69–90.49%	51.85	33.99–69.26%	1.662
<963.5	83.33	66.44–92.66%	51.85	33.99–69.26%	1.731
<1,017	86.67	70.32–94.69%	51.85	33.99–69.26%	1.800
<1,027	86.67	70.32–94.69%	48.15	30.74–66.01%	1.671
<1,042	90.00	74.38–96.54%	48.15	30.74–66.01%	1.736
<1,066	90.00	74.38–96.54%	44.44	27.59–62.69%	1.620
<1,075	90.00	74.38–96.54%	40.74	24.51–59.27%	1.519
<1,087	93.33	78.68–98.82%	40.74	24.51–59.27%	1.575
<1,116	93.33	78.68–98.82%	37.04	21.53–55.77%	1.482
<1,210	93.33	78.68–98.82%	33.33	18.64–52.18%	1.400
<1,307	93.33	78.68–98.82%	29.63	15.85–48.48%	1.326
<1,341	93.33	78.68–98.82%	25.93	13.17–44.68%	1.260
<1,411	93.33	78.68–98.82%	22.22	10.61–40.76%	1.200
<1,495	93.33	78.68–98.82%	18.52	8.181–36.70%	1.145
<1,541	93.33	78.68–98.82%	14.81	5.916–32.48%	1.096
<1,577	93.33	78.68–98.82%	11.11	3.852–28.06%	1.050
<1,632	96.67	83.33–99.83%	11.11	3.852–28.06%	1.088
<1,894	96.67	83.33–99.83%	7.407	1.316–23.37%	1.044
<2,368	100.0	88.65–100.0%	7.407	1.316–23.37%	1.080
<3,721	100.0	88.65–100.0%	3.704	0.1900–18.28%	1.038

Abbreviations: AUC, area under the curve; CI, confidence interval; ROC, receiver operating characteristic.

Levels of granzyme B, CXCL9, CXCL10, IL12p70, and CCL18 were measured using the ELLA Protein Simple Plex Immunoassay (Protein Simple, San Jose, CA). Samples were diluted in provided sample diluent and run according to the manufacturer's instructions. Matrix metalloproteinase 12, galectin-9, MIP3β, and IL-2 receptor were detected and quantified using the ProCarta Multiplex Immunoassay (Thermo Fisher Scientific, Waltham, MA). Serum was diluted in provided universal assay buffer and incubated for 2 hours at room temperature. Assay plates were read on a Luminex 200 Instrument (Luminex, Austin, TX). The concentration of each analyte was extrapolated from the antigen standard curve; concentrations are expressed as pg/ml. To calculate the inflammatory signal, raw pg/ml values for each analyte (e.g., CXCL9, CXCL10, and MIP3) were added together to generate a single value for each collection (i.e., for each patient and time point).

Statistical analysis

Posthoc analyses of the phase two clinical trial were performed to further characterize the patients achieving F-VASI50 at week 24. The efficacy outcome of F-VASI50 at week 24 was used to stratify patients into subgroups for further analysis.

Broad proteomics was explored for patterns that may elucidate mechanisms connecting ruxolitinib cream treatment to clinical response. We conducted a two-sample *t*-test comparing proteomic differences between patients who achieved the primary endpoint of F-VASI50 at week 24 (group 1) and those who did not (group 2). Fold changes, raw *P*-values, and Benjamini–Hochberg false discovery rate–corrected *P*-values are reported for proteins with raw *P*-values < 0.05

Additional statistical analysis (receiver operating characteristic, area under the curve) to test the diagnostic ability of the inflammatory signal was further tested by comparing group 1 (designated as the test group) with group 2 (designated as the control group) using the Wilson/Brown hybrid method (Brown et al., 2001). The area under the curve for the receiver operating characteristic curve was calculated using GraphPad Prism (San Diego, CA) by generating a curve created by nonlinear regression. The sensitivity and specificity table was generated using different cutoff values for the inflammatory signal level. Area under the curve/receiver operating characteristic results were used to calculate many pairs of sensitivity and specificity, and these values and their corresponding 95% confidence interval values were tabulated (Table 6).

Data availability statement

Incyte (Wilmington, DE) is committed to data sharing that advances science and medicine while protecting patient privacy. Qualified external scientific researchers may request anonymized datasets owned by Incyte for the purpose of conducting legitimate scientific research. Researchers may request anonymized datasets from any interventional study (except phase 1 studies) for which the product and indication have been approved on or after January 1, 2020 in at least one major market (e.g., United States, European Union, Japan). Data will be available for request after the primary publication or 2 years after the study has ended. Information on Incyte's clinical trial data–sharing policy and instructions for submitting clinical trial data requests are available at https://www.incyte.com/Portals/0/Assets/Compliance%20and%20Transparency/clinical-trial-data-sharing.pdf?ver=2020-05-21-132838-960

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CONFLICT OF INTEREST

MDH and FIK were employees and shareholders of Incyte at the time of the study. MDH is a current employee and shareholder of DermTech. FIK is a current employee and shareholder of Arena Pharmaceuticals. BR, EB, KS, and KB are employees and shareholders of Incyte. JEH has served as a consultant for AbbVie, Aclaris Therapeutics, BiologicsMD, EMD Serono, Genzyme/Sanofi, Janssen, Pfizer, Rheos Medicines, Sun Pharmaceutcals, TeVido BioDevices, The Expert Institute, 3rd Rock Ventures, Villaris Therapeutics, Almirall, AnaptysBio, Avita, Boston Pharma, BridgeBio, Cogen Therapeutics, Dermavant, Frazier Management, Granular Therapeutics, Methuselah Health, Pandion, Platelet Biogenesis, Sonoma Biotherapeutics, Temprian Therapeutics, Twi Biotech, and Vimela; has served as an investigator for Aclaris Therapeutics, Celgene, Dermira, EMD Serono, Genzyme/Sanofi, Incyte, LEO Pharma, Pfizer, Rheos Medicines, Stiefel/GlaxoSmithKline, Sun Pharmaceuticals, TeVido BioDevices, and Villaris Therapeutics; holds equity in Aldena Therapeutics, NIRA Biosciences, Rheos Medicines, TeVido BioDevices, and Villaris Therapeutics; is a scientific founder of Aldena Therapeutics, NIRA Biosciences, and Villaris Therapeutics; and has patents pending for IL-15 blockade for treatment of vitiligo, Jak inhibition with light therapy for vitiligo, and CXCR3 antibody depletion for treatment of vitiligo. PG has served as a consultant for Aclaris Therapeutics, Clarify Medical, DermaForce, Incyte, Proctor & Gamble, and Versicolor Technologies and as a principal investigator for Aclaris Therapeutics, Allergan/SkinMedica, Clinuvel Pharmaceuticals, Incyte, Johnson & Johnson, L'Oreal, Merz Pharma, Pfizer, Thync Global, and VT Cosmetics. DR has received honoraria as a consultant for AbbVie, Abcuro, AltruBio, Arena, Boehringer Ingelheim, Bristol Myers Squibb, Celgene, Concert, CSL Behring, Dermavant Sciences, Dermira, Incyte, Janssen, Kyowa Kirin, Lilly, Nektar, Novartis, Pfizer, RAPT, Recludix, Regeneron Pharmaceuticals, Revolo Biotherapeutics, Sanofi, Sun Pharmaceuticals, UCB, and Viela Bio; has received research support from AbbVie, Amgen, Bristol Myers Squibb, Celgene, Dermira, Galderma, Incyte, Janssen, Lilly, Merck, Novartis, Pfizer, and Regeneron Pharmaceuticals; and has served as a paid speaker for AbbVie, Amgen, Celgene, Janssen, Lilly, Novartis, Pfizer, Regeneron Pharmaceuticals, and Sanofi.

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AUTHOR CONTRIBUTIONS

Conceptualization: MDH, FIK, KS, KB, JEH, PG, DR; Data Curation: MDH, BR, EB; Formal Analysis: MDH, FIK, BR, EB, KB, JEH, PG, DR; Validation: MDH, BR, EB, KS; Writing - Original Draft Preparation: MDH, FIK, KB, JEH, PG, DR; Writing - Reviewing and Editing: MDH, FIK, BR, EB, KS, KB, JEH, PG, DR

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