SUPPLEMENTARY APPENDIX

To: Alessandro M Vannucchi et al. Compassionate Use of JAK1/2 Inhibitor Ruxolitinib for Severe Covid-19.

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RUXO-COVID Study Group Participants.

Participant	Institution
Alessandro Maria Vannucchi	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Benedetta Sordi	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Alessandro Morettini	Internal Medicine Unit 2, AOU Careggi, Florence, Italy
Carlo Nozzoli	Internal Medicine Unit 1, AOU Careggi, Florence, Italy
Loredana Poggesi	Internal Medicine Unit 3, AOU Careggi, Florence, Italy
Filippo Pieralli	Intermediate Care Unit Covid-19, AOU Careggi, Florence, Italy
Alessandro Bartoloni	Infectious and Tropical Diseases Unit, AOU Careggi, Florence, Italy
Alessandro Atanasio	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Filippo Miselli	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Chiara Paoli	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Giuseppe G. Loscocco	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Andrea Fanelli	Internal Medicine Unit 2, AOU Careggi, Florence, Italy
Ombretta Para	Internal Medicine Unit 1, AOU Careggi, Florence, Italy
Andrea Berni	Internal Medicine Unit 3, AOU Careggi, Florence, Italy
Irene Tassinari	Intermediate Care Unit Covid-19, AOU Careggi, Florence, Italy
Lorenzo Zammarchi	Infectious and Tropical Diseases Unit, AOU Careggi, Florence, Italy
Laura Maggi	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Alessio Mazzoni	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Valentina Scotti	Internal Medicine Unit 2, AOU Careggi, Florence, Italy
Danilo Malandrino	Internal Medicine Unit 3, AOU Careggi, Florence, Italy
Fabio Luise	Intermediate Care Unit Covid-19, AOU Careggi, Florence, Italy
Giovanni Millotti	Infectious and Tropical Diseases Unit, AOU Careggi, Florence, Italy
Sara Bencini	Cytometry and Immunotherapy Diagnostic Center (CDCI), AOU Careggi, Florence, Italy
Duccio Fantoni	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Giorgia Falchetti	Internal Medicine Unit 2, AOU Careggi, Florence, Italy
Miriam Borella	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Enrica Ravenda	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Manuela Capone	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy

Marie Pierre Piccinni	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Francesco Annunziato	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy; Cytometry and Immunotherapy Diagnostic Center (CDCI), AOU Careggi, Florence, Italy
Paola Guglielmelli	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Francesco Mannelli	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Giacomo Coltro	Center Research Innovation of Myeloproliferative Neoplasms (CRIMM), SOD Hematology, University of Florence and AOU Careggi, Florence, Italy
Benedetta Peruzzi	Cytometry and Immunotherapy Diagnostic Center (CDCI), AOU Careggi, Florence, Italy
Roberto Caporale	Cytometry and Immunotherapy Diagnostic Center (CDCI), AOU Careggi, Florence, Italy
Lorenzo Cosmi	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Francesco Liotta	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Letizia Lombardelli	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Federica Logiodice	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Anna Vanni	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Lorenzo Salvati	Department of Clinical and Experimental Medicine, University of Florence, Florence, Italy
Chiara Lazzeri	Department of Neuromuscoloskeletric and sense organs, AOU Careggi, Florence, Italy
Manuela Bonizzoli	Department of Neuromuscoloskeletric and sense organs, AOU Careggi, Florence, Italy
Adriano Peris	Department of Neuromuscoloskeletric and sense organs, AOU Careggi, Florence, Italy
Giovanni Cianchi	Department of Neuromuscoloskeletric and sense organs, AOU Careggi, Florence, Italy
Alberto Bosi	SOD Hematology, AOU Careggi, Florence, Italy
Michela Pucatti	Hospital Pharmacy, AOU Careggi, Florence, Italy
Fontanari Paolo	Department of Anesthesia and Reanimation, AOU Careggi, Florence, Italy
Silvia Benemei	Ethics Committee AVC, AOU Careggi, Florence, Italy
Marco Matucci Cerinic	Ethics Committee AVC, AOU Careggi, Florence, Italy
Lucia Turco	Health management, AOU Careggi, Florence, Italy

Supplementary Methods

Immunophenotyping and intracellular staining by multiparametric flow cytometric analysis

All flow cytometric analysis were performed following published guidelines[1]. Samples were acquired on a BD LSR II flow cytometer or FACS Canto II (BD Biosciences). List of all fluorochrome-conjugated mAbs is reported in Table X (supplementary). Different leukocytes cell subsets were identified by staining lineage surface markers on whole blood, followed by red blood cells lysis prior to FACS analysis. Intracellular staining was performed on PBMNC (peripheral blood mononuclear cells), obtained following density gradient centrifugation of fresh blood samples. In details, PBMNC were fixed in formaldehyde 2%, and stained with fluorochrome-conjugated mAbs in presence of saponin 0,5%. For the evaluation of intracellular cytokines production, PBMNC were previously polyclonally stimulated for 5 hours with PMA and ionomycin, the last 3 in presence of brefeldin A[2]. Evaluation of Ki67 expression was performed following the manufacturer's instructions of anti-Human Foxp3 Staining Set (eBioscience) for fixing and staining details[3].

Determination of serum cytokines and chemokines concentration with bead-based multiplex immunoassays

Sera were collected and stored at -20°C. The quantitative determination of IL-1beta, IL-1Ralpha, IL-2, IL-4, IL-5, IL-6, IL-7 IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IFN-gamma, TNF-alpha, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES, eotaxin, MIP-1-alpha, and MIP-1-beta in the serum was performed by using a bead-based multiplex immunoassay (Biorad Laboratories) and the Bioplex 200 system (Biorad Laboratories) as previously described[2].

In brief, in a 96-well filter plate (Bio-Rad) 50µl of each serum sample were added to 50 µl of antibody-conjugated beads directed against the cytokines listed above (Bio-Rad). After a 30-min incubation, the plate was washed and 25 µl of biotinylated anti-cytokine antibody solution were added to each well

before another 30-min incubation. The plate was then washed and 50 µl of streptavidin-conjugated phycoerithrin were added to each well. After a final wash, each well was resuspended with 125 µl of assay buffer (Bio-Rad) and analyzed by Bioplex 200 (Biorad Laboratories, Hercules, CA).

Standard curves were derived from various concentrations of the cytokine standards and followed the same protocol as the serum samples. The concentration of the 27 cytokines (pg/ml) in each serum sample was calculated using the Bioplex 200 software.

Table S1. List of fluorochrome mAbs used for flow cytometric analysis

Marker	Clone	Flurochrome	Supplier
CCR7	150503	Horizon™ V450	BDBioscience
CD16	3G8	FITC PerCP-cy5.5	BDBioscience
CD19	SJ25C1	APC	BDBioscience
CD3	UCHT1	Pacific Blue™ Horizon™ V450	BDBioscience
CD3	SK7	PE	BDBioscience
CD4	SK3	PE-Cy™7 PerCP	BDBioscience
CD45	2D1	Horizon™ V500	BDBioscience
CD45RA	L48	FITC	BDBioscience
CD56	MY31	PE	BDBioscience
CD56	NCAM16.2	PE	BDBioscience
CD56	B159	APC	BDBioscience
CD8	SK1	APC-Cy™7 APC APC-H7	BDBioscience
HLA-DR	L243	FITC Horizon™ V450	BDBioscience
IFN-γ	45-15	APC	Miltenyi Biotech
IFN-γ	25723.11	FITC	BDBioscience
TNF-α	6401.1111	FITC	BDBioscience
Ki67	20Raj1	PE	Thermo Fisher Scientific
CD15	MMAC	FITC	BDBioscience
CD123	9F5	PE	BDBioscience
CD64	10,1	PerCP-cy5.5	BDBioscience
CD13	L138	PE-Cy™7	BDBioscience
CD11b	D12	APC	BDBioscience
CD14	MϕP9	FITC	BDBioscience
CD33	P676	PE-Cy™7	BDBioscience
CD300	UP-H2	APC	BDBioscience
CD1c	AD5-8E7	PE	Miltenyi Biotech
CD141	1A4	APC	BDBioscience
CD66b	G10F5	Horizon™ V450	BDBioscience

Table S2. Baseline predictors of clinical improvement

Variable	Hazard Ratio (95% CI)
Age, per years	1.0 (0.9-1.0)
<60 years	-
60 to <70 years	3.1 (0.6-14.9)
70 to <80 years	1.1 (0.3-4.3)
≥ 80 years	1.5 (0.4-5.4)
Female sex	0.8 (0.4-1.7)
Duration of symptoms prior to ruxolitinib treatment	0.9 (0.9-1.0)
Comorbidities	
Neoplasia	0.5 (0.2-1.2)
Hypertension	0.5 (0.2-1.2)
Diabetes mellitus	0.4 (0.2-1.3)
Cardio vascular diseases	1.0 (0.5-2.1)
Pulmonary diseases	1.2 (0.5-2.7)
Neurological diseases	0.8 (0.4-1.6)
Autoimmune diseases	1.6 (0.5-4.8)
Laboratory tests	
Lymphocytes, per x10^9/L	0.9 (0.4-2.4)
C-reactive protein, per mg/l	1.0 (0.9-1.0)
D-Dimer, per ng/ml	1.0 (0.9-1.0)
Ferritin, per mg/l	1.0 (0.9-1.1)

CI, 95% Confidence Interval

Table S3: Longitudinal evaluation of leukocyte subsets in COVID-19 patients during treatment with ruxolitinib.

Cell Population	Controls	T0 (cells/µl)	T7 (cells/µl)	T14 (cells/µl)
Neutrophils	3.79 (±1.17)	4,96 (±2.75)	4.18 (±2.55)	4.29 (±2.38)
Lymphocytes	1.83 (±0.59) ***	0.96 (±0.45)	1.05 (±0.53)	1.25 (±0.62) *
Monocytes	0.53 (±0.10) *	0.37 (±0.20)	0.36 (±0.18)	0.46 (±0.16) *
Eosinophils	0.19 (±0.09) ***	0.03 (±0.07)	0.07 (±0.08) *	0.08 (±0.1) *
Basophils	0.03 (±0.02)	0.02 (±0.03)	0.03 (±0.07)	0.03 (±0.01)
Myeloid DC	0.0133 (±0.0045) ***	0.0026 (±0.0018)	0.0049 (±0.0032) *	0.0093 (±0.0042) ***
Plasmacytoid DC	0.0106 (±0.0059)	0.0064 (±0.01)	0.0043 (±0.0038)	0.0110 (±0.011) **

* p<0.05 versus T0

** p<0.01 versus T0

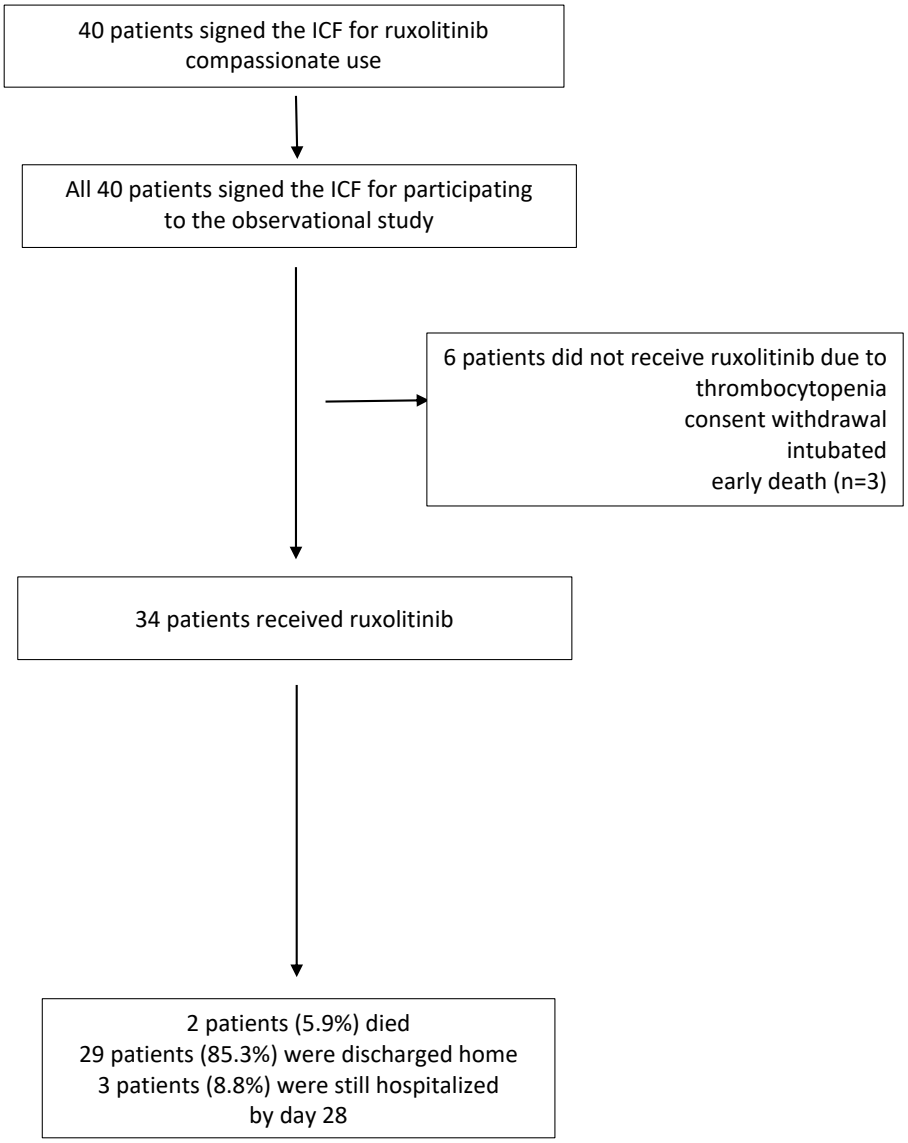
*** p<0.001 versus T0

Table S4: Longitudinal evaluation of serum cytokine levels in COVID-19 patients during treatment with ruxolitinib.

Cytokine	Controls (pg/ml)	T0 (pg/ml)	T7 (pg/ml)	T14 (pg/ml)
IL-1β	0.088 (±0.022) *	0.57 (±0.39)6.5	0.63 (±0.31)	0.139 (±0.038) ***
IL-1rα	24,6 (±17.1)	2117.37 (±4015.37)86	2024.35 (±4441.62)	288.47 (±537.79)
IL-2	0.14 (±0.17) **	3.70 (±1.97)9.3	3.27 (±1.91)	6.58 (±23.84)
IL-4	0.40 (±0.37) **	3.52 (±2.04)8.8	3.22 (±1.81)	0.87 (±0.51) ***
IL-5	Undetected	5.66 (±7.80)	3.27 (±5.43)	0.78 (±1.32) *
IL-6	0.05 (±0.05) *	4.48 (±3.28)89.6	2.1 (±1.49) *	0.48 (±0.55) ***
IL-7	Undetected	Undetected	Undetected	Undetected
IL-8	1.88 (±1.46) *	25.95 (±17.53)13.7	32.92 (±31.78)	7.06 (±6.75) ***
IL-9	0.32 (±0.41)	4.12 (±6.45)13.8	3.45 (±3.66) *	0.71 (±0.75) **
IL-10	1.18 (±0.13) ***	8.47 (±2.70)7.2	5.45 (±1.47) ***	1.27 (±0.25) ***
IL-12	0.51 (±0) ***	3.50 (±1.14)7	3.09 (±0.91)	0.78 (±0.27) ***
IL-13	0.227 (±0.277) *	2.56 (±1.73)11.6	2.02 (±1.31)	0.32 (±1.24) ***
IL-15	Undetected	Undetected	Undetected	Undetected
IL-17A	1.35 (±0.53) *	10.31 (±7.10)7.6	9.41 (±4.10)	2.44 (±0.98) ***
Eotaxin	19.09 (±18.11) *	113.52 (±73.59)5.9	124.68 (±84.67)	39.42 (±27.02) ***
FGF	8.52 (±2.76) ***	45.78 (±8.62)5.5	43.10 (±9.88)	13.08 (±5.00) ***
G-CSF	Undetected	Undetected	Undetected	Undetected
GM-CSF	Undetected	Undetected	Undetected	Undetected
IFN-γ	Undetected	2.13 (±3.64)	2.09 (±5.23)	0.10 (±0.25) *
IP10	53.61 (±57.53) **	4689.77 (±3134.34)87.5	1938.24 (±2044.08) **	153.37 (±91.25) ***
MCP1	13.48 (±10.41)	163.78 (±190.66)54.3	119.50 (±178.12)	18.38 (±19.36) **
MIP1A	0.41 (±0.41) **	5.12 (±2.95)12	7.56 (±4.50)	1.99 (±1.11) **
MIP1B	28.14 (±9.78) ***	114.44 (±21.15)4.1	124.09 (±28.59)	34.35 (±4.05) ***
PDGF	1125.53 (±808.97)	2481.46 (±1648.44)2.2	3110.84 (±2268.37)	832.84 (±495.67) ***
Rantes	2981.05 (±2146.47)	12729.1 (±17247.1)4.3	12910.8 (±7519.04)	3747.64 (±1726.37) *
TNF-α	4.92 (±1.12) **	39.03 (±20.82)9.5	33.87 (±13.07)	8.09 (±1.98) ***
VEGF	Undetected	Undetected	Undetected	Undetected

* $p<0.05$ versus T0; ** $p<0.01$ versus T0; *** $p<0.001$ versus T0

Figure S1. Patient Disposition.



ICF= Informed Consent Form

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