Letter to the Editor

Broad spectrum of autoantibodies in patients with Wiskott-Aldrich syndrome and X-linked thrombocytopenia

To the Editor:

Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT) are allelic diseases caused by mutations of the *WAS* gene.[1] Autoimmune manifestations (especially cytopenias, inflammatory bowel disease, vasculitis, arthritis, and IgA nephropathy) affect between 24% and 72% of patients with WAS in various series, with important implications on quality of life and survival.[2] Although patients with XLT do not have autoimmune manifestations at diagnosis, some of them can have autoimmunity over time.[3]

To investigate in greater detail and compare the degree of immune dysregulation in WAS and XLT, we have studied 17 patients with WAS and 10 patients with XLT. The clinical and laboratory features of the patients are reported in Table I.

Plasma samples from patients with WAS/XLT were diluted 1:100 in PBS and 100 μL of the dilution was incubated in duplicate with an autoantigen proteomic array (University of Texas Southwestern Medical Center, Genomic and Microarray Core Facility),[4] which includes 67 and 77 self-antigens, respectively, to analyze the frequency, antigen specificity, and isotype composition of autoantibodies. Plasma from 6 healthy control subjects and 5 patients with systemic lupus erythematous served as negative and positive controls, respectively. The arrays were then incubated with Cy3-labeled anti-human IgG and Cy5-labeled anti-human IgA antibodies, respectively, to define the IgG or IgA isotype specificity of the autoantibodies. Tiff images were generated by using the GenePix 4000B scanner (Molecular Devices, Sunnyvale, Calif) with laser wavelengths of 532 nm (for Cy3) and 635 nm (for Cy5) and analyzed with GenePix Pro 6.0 software. Net fluorescence intensity (defined as the spot minus background fluorescence intensity) data obtained from duplicate spots were averaged. Data were normalized as follows. Across all samples, the immunoglobulin-positive controls (IgG or IgA) were averaged, and the positive controls in each sample were divided by the averaged positive control, generating a normalization factor for each sample. Each signal was than multiplied by the normalization factor for each block (sample). For each antigen, values from healthy donor samples ($n \geq 3$) were averaged. For each sample, ratios were then calculated between the value in the sample and the average of values in healthy donors plus 2 SDs, thus defining relative autoantibody reactivity (RAR) of the sample. RAR values of greater than 1 were considered positive. A heat map of the ratio values was generated by using MultiExperiment Viewer software (DFCI, Boston, Mass). Significant differences in autoantibody signal between groups were assessed by using Significance Analysis of Microarrays (Stanford University Laboratories, Stanford, Calif) with a false discovery rate of less than 1%.

As shown in Fig 1, *A* and *B*, the presence of at least 1 positive IgG and IgA autoantibody was documented in the vast majority of patients with WAS and those with XLT. Autoantibody levels that were significantly increased in patients with WAS and those with XLT compared with those in healthy donors are shown in Fig 1, *C* and *D*.

Samples were considered multireactive if they contained autoantibodies to at least 20% of the self-antigens represented on the array. Multireactivity of IgG autoantibodies was observed in 16 (59.2%) of 27 patients, specifically in 11 of 17 samples from patients with WAS and 5 of 10 samples from patients with XLT (see Fig E1 in this article's Online Repository at www.jacionline.org).

Multireactivity of IgA autoantibodies was observed in 12 (46.1%) of 26 patients, in particular 7 of 16 samples from patients with WAS and 5 of 10 samples from patients with XLT (see Fig E1). Patients with autoantibody multireactivity had significantly higher serum IgA levels compared with patients with reactivity to less than 20% of the self-antigens tested, and a similar trend was observed for serum IgG levels (see Fig E2 in this article's Online Repository at www.jacionline.org). Self-antigens to which autoantibodies were demonstrated in more than 20% of patients with WAS/XLT were defined as "common autoantigens." The 25 most common IgG and IgA autoantibodies are reported in Fig E3 in this article's Online Repository at www.jacionline.org. Of note, 9 (36%) of the 25 top most common autoantigens were the target of both IgG and IgA autoantibodies (mitochondrial antigen, fibrinogen IV, entactin, M2 antigen, myosin, elastin, LC1, SRP54, sn-RNP-68, and Scl-70). By using RAR for semiquantitative analysis of signal intensity, IgG autoantibodies to 2 common antigens (fibrinogen IV and mitochondrial antigen) were present at higher levels in patients with WAS and those with XLT versus healthy control subjects (see Fig E3).

Multiple immunologic abnormalities have been identified that might account for immune dysregulation in patients with WAS,[5] including impaired function of regulatory T and regulatory B cells, defective apoptosis, abnormalities of the distribution and diversity of T and B lymphocytes, and defective function of T and natural killer cells, resulting in impaired clearance of pathogens and persistent inflammation. Moreover, Wiskott-Aldrich syndrome protein (WASP)–deficient plasmacytoid dendritic cells are hyperresponsive to Toll-like receptor 9 stimulation and produce high amounts of type 1 interferon, which might also contribute to autoimmunity.[6] More recently, we and others have identified B-cell autonomous effects of WASP deficiency that are likely to play a critical role in the autoimmunity of the disease.[7-9] These include (1) hyperresponsiveness of WASP-deficient B cells to stimulation through the B-cell receptor and Toll-like receptors; (2) accumulation of B lymphocytes with a characteristic phenotype ($CD21^{low}CD38^{low}$), which is indicative of a type 1 interferon signature and a marker of self-reactivity; (3) preferential use of immunoglobulin variable genes that are enriched in patients with autoimmune disease and decreased somatic hypermutation; (4) increased release of immature B cells from the bone marrow to the periphery; (5) increased levels of B cell–activating factor of the TNF family serum; and (6) decreased regulatory B-cell function. In our series increased levels of B cell–activating factor of the TNF family serum were found not only in patients with WAS but also in those with XLT (Table I).

To our knowledge, our study represents the first attempt at extensively analyzing the frequency and diversity of autoantibodies in patients with WAS versus those with XLT. Our data indicate that biological signs of immune dysregulation are a

TABLE I. Molecular, immunologic, and clinical characteristics of patients

Patient ID	Age	Mutation	IgG (mg/dL [reference range])	IgA (mg/dL [reference range])	IgM (mg/dL [reference range])	IgE (kU/L [reference range])	BAFF (pg/mL [reference range])	Autoimmunity	Autoantibodies (clinical testing)
XLT 18	19 y	p. L39P	664 (639-1,344)	241 (70-312)	**14** (34-210)	**>5,000** (0-200)	**2,400** (469-1,104)	—*†	—
XLT 19	22 y	p. L39P	954 (639-1,344)	221 (70-312)	**15** (34-210)	**374** (0-200)	**3,300** (469-1,104)	—*	ASMA
XLT 22	19 y	p. P58 R	741 (639-1,344)	128 (70-312)	66 (34-210)	109 (0-200)	NA	—	—
XLT 23	16 y	p. P58 R	1,220 (639-1,344)	144 (70-312)	149 (34-210)	171 (0-200)	**2,250** (469-1,104)	—	—
XLT 33	8 y	p.T45 K	914 (639-1,344)	266 (70-312)	**34** (34-210)	31 (0-200)	**1,550** (469-1,104)	—*	ANA, ANCA, ASMA
XLT 37	15 y	p. D77 G	1,000 (639-1,344)	**605** (70-312)	**20** (34-210)	**374** (0-200)	**1,200** (469-1,104)	—	ANA
XLT 38	11 y	p. D77 G	950 (639-1,344)	211 (70-312)	**20** (34-210)	83 (0-500)	**2,100** (469-1,104)	—	ANA
XLT 40	17 y	p. I481 N	**1,360** (639-1,344)	**396** (70-312)	43 (34-210)	13 (0-200)	NA	—	—
XLT 51	5 y	p. V75 M	950 (600-1,500)	110 (50-150)	40 (22-100)	27 (0-200)	450 (469-1,104)	—	—
XLT 52	19 mo	p.V51 L	680 (400-1,300)	65 (20-230)	57 (30-120)	**73** (0-30)	**2,550** (469-1,104)	—	—
WAS 3	4 mo	p. F74S	NA	NA	NA	NA	**9,900** (469-1,104)	NA	ND
WAS 9	2 y	p. W64 R	1,090 (400-1,300)	210 (20-230)	119 (30-120)	NA	**2,700** (469-1,104)	—	ANA
WAS 17	5 y	c.777+1g > c	**491** (600-1,500)	**431** (50-150)	44 (22-100)	**383** (0-200)	NA	—	—
WAS 21	1 mo	p.Q255Pfs*5	**617** (700-1,300)	38 (6-50)	20 (15-70)	69 (0-30)	750 (469-1,104)	—	—
WAS 25	5 y	p. P362Qfs*132	**533** (600-1,500)	**44** (50-150)	70 (22-100)	170 (0-200)	NA	—	Plt, TPO
WAS 27	3 y	p. W64 R	1,750 (600-1,500)	**594** (50-150)	**28** (30-120)	**770** (0-200)	NA	AIHA, IBD	Coombs
WAS 28	5 y	p. W64 R	**246** (600-1,500)	**17** (50-150)	27 (22-100)	NA	NA	AIHA, IBD, vasculitis	Coombs
WAS 30	3 y	p. V106Cfs*15	**246** (600-1,500)	**17** (50-150)	**27** (30-120)	NA	**6300** (469-1,104)	Vasculitis	PL, Plt
WAS 33	9 y	p. E67Efs*4	968 (639-1,344)	**526** (70-312)	**20** (34-210)	35 (0-200)	NA	IBD	—
WAS 35	20 mo	p. D495Mfs*98	483 (400-1,300)	45 (20-230)	**5** (30-120)	NA	NA	—	Coombs, ANCA
WAS 37	8 mo	p. P110Lfs*13	924 (300-1,500)	71 (16-100)	33 (25-115)	**128** (0-30)	**2,200** (469-1,104)	—	ND
WAS 38	1 y	p. G424Afs*20	749 (300-1,500)	**225** (16-100)	28 (25-115)	NA	**5,100** (469-1,104)	NA	ND
WAS 40	10 mo	p. R86H	NA	NA	NA	NA	**2,600** (469-1,104)	—	ANA, Coombs, Plt
WAS 42	11 mo	p.P373Hfs*72	898 (300-1,500)	82 (16-100)	59 (25-115)	**370** (0-30)	**1,200** (469-1,104)	—	—
WAS 43	2 y	p. V473Gfs*14	1,784 (400-1,300)	**233** (20-230)	111 (30-120)	NA	**10,000** (469-1,104)	—	ND
WAS 49	2 y	c.559+5g>a	NA	NA	NA	NA	**2,400** (469-1,104)	—	TPO
WAS 50	3 y	c.559+5g>a	NA	NA	NA	NA	**2,000** (469-1,104)	—	TPO
WAS 60	48 y	c.559+5g>a	1,068 (639-1,344)	**881** (70-312)	**15** (34-210)	**440** (0-200)	NA	—	PL

Values in boldface are outside of age-matched reference values (shown in parentheses).
AIHA, Autoimmune hemolytic anemia; *ANA*, anti-nuclear antibodies; *ANCA*, anti-neutrophil cytosplasmic antibodies; *ASMA*, anti–smooth muscle antibodies; *BAFF*, B cell–activating factor of the TNF family; *IBD*, inflammatory bowel disease; *NA*, not available; *ND*, not done; *PL*, anti-phospholipid antibodies; *Plt*, anti-platelet antibodies; *TPO*, anti-thyroid peroxidase antibodies.
*This patient had vasculitis later in life.
†This patient had arthritis later in life.

characteristic feature of patients with loss-of-function mutations of the *WAS* gene, irrespective of the severity of the clinical phenotype. This biological signature of immune dysregulation might set the stage for progressive development of clinical manifestations of autoimmunity also in patients with XLT. Consistent with this, 3 of the patients with XLT included in this study (XLT 18, XLT 19, and XLT 33) had cutaneous vasculitis later in the course of their disease, and 1 of them (XLT 18) also had arthritis, a pattern that has been reported in several other patients with *bona fide* XLT.[9] These data strongly suggest that XLT should not be considered a distinct disease entity but rather part of the clinical spectrum of WAS. Prospective longitudinal studies are needed to assess whether differences in the amount, diversity, and avidity of autoantibodies produced are predictive of development of clinical manifestations of autoimmunity in patients with XLT/WAS.

Elena Crestani, MD[a]*
Stefano Volpi, MD[a,b]*
Fabio Candotti, MD[c]
Silvia Giliani, PhD[d]
Lucia Dora Notarangelo, MD[e]
Julia Chu, MD[f]
Juan Carlos Aldave Becerra, MD[g]
David Buchbinder, MD[h]
Janet Chou, MD[a]
Raif S. Geha, MD[a]
Maria Kanariou, MD[i]
Alejandra King, MD[j]
Cinzia Mazza, PhD[d]
Daniele Moratto, PhD[d]
Robert Sokolic, MD[c]
Elizabeth Garabedian, RN, MSLS[c]
Fulvio Porta, MD[e]
Maria Caterina Putti, MD[k]
Rima H. Wakim, MD[l]
Erdyni Tsitsikov, PhD[m]
Sung-Yun Pai, MD[f]
Luigi D. Notarangelo, MD[a]

From the Divisions of [a]Immunology and [f]Hematology-Oncology and [m]the Department of Laboratory Medicine, Boston Children's Hospital, Boston, Mass; [b]the Department of Neuroscience, Rehabilitation, Ophthalmology, Genetics, Maternal and Child Health, University of Genoa, Genoa, Italy; [c]the Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Md; [d]"Angelo Nocivelli" Institute for Molecular Medicine, University of

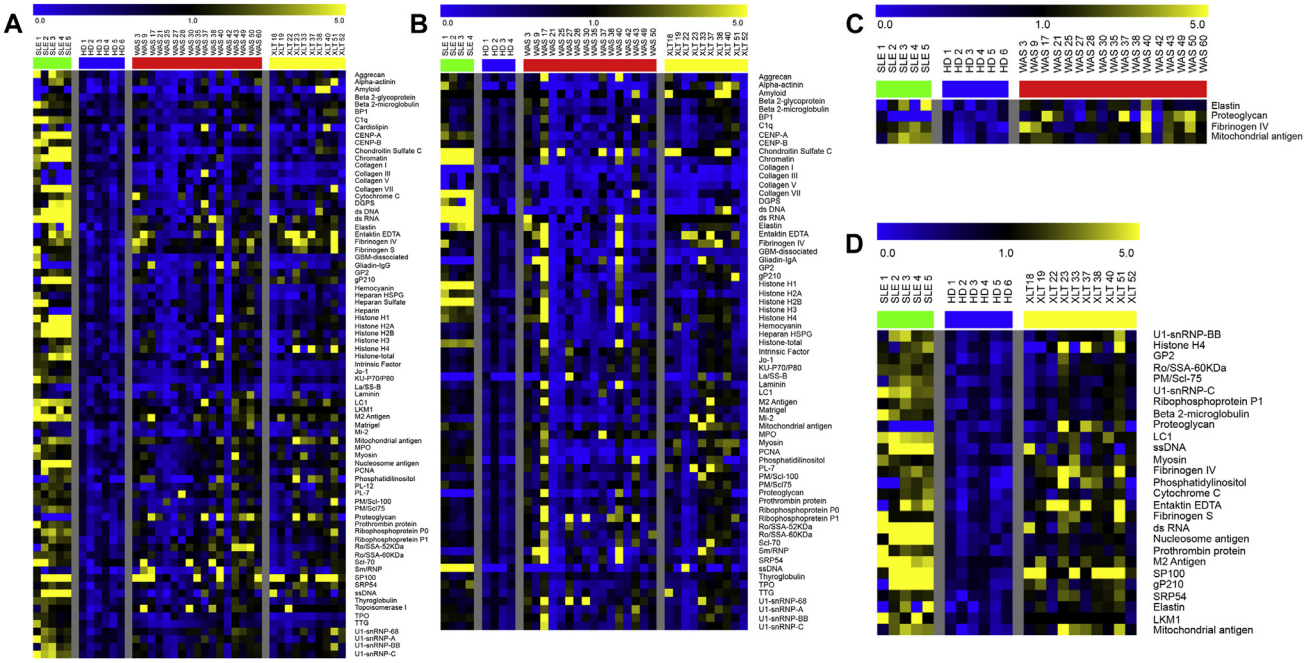

FIG 1. **A** and **B,** Heat map of IgG (Fig 1, *A*) and IgA (Fig 1, *B*) autoantibodies in patients with XLT and those with WAS. For each self-antigen, a colorimetric representation of RAR in each sample is shown according to the scale depicted at the top. **C** and **D,** Autoantibodies with significantly increased reactivity in patients with WAS and those with XLT, respectively, compared with those in healthy donors *(HD)*.

Brescia, Brescia, Italy; [e]the Pediatric Hematology Oncology Unit, Spedali Civili, Brescia, Italy; [g]the Division of Allergy and Clinical Immunology, Rebagliati Martins National Hospital, Lima, Peru; [h]the Division of Pediatric Hematology, Children's Hospital Orange County, University of California at Irvine, Irvine, Calif; [i]the Department of Immunology, "Aghia Sophia" Children's Hospital, Athens, Greece; [j]the Division of Pediatric Immunology, Hospital Luis Calvo Mackenna, Santiago, Chile; [k]the Clinic of Pediatric Hematology-Oncology, Department for Woman and Child Health, University Hospital, Padua, Italy; and [l]the Department of Pediatrics and Adolescent Medicine, American University of Beirut, Beirut, Lebanon. E-mail: luigi.notarangelo@childrens.harvard.edu.

*These authors contributed equally to this work.

Supported by a grant from the National Heart, Lung, and Blood Institute/National Institutes of Health (grant 5P01HL059561-13 to L.D.N.); an educational grant (5T32AI007512) from the National Institute of Allergy and Infectious Diseases (to E.C. [Dr Raif S. Geha, principal investigator]); an educational grant from the National Heart, Lung and Blood Institute/National Institutes of Health (grant 5T32HL007574-33 to J.C.); and a grant from the UNIL-CHUV (CGRB 29583 to F.C.).

Disclosure of potential conflict of interest: F. Candotti has received a grant from University of Lausanne-Centre hospitalier universitaire vaudois and is employed by Centre hospitalier universitaire vaudois. J. Chu has received a grant from the National Institutes of Health (NIH). J. Chou is employed by Boston Children's Hospital and has received grants from the NIH and the Jeffrey Modell Foundation. F. Porta has received payment for lectures from Pfizer. S.-Y. Pai has received a grant from Translational Investigator Service, is employed by Boston Children's Hospital, and has a grant pending from the National Heart, Lung, and Blood Institute. L. D. Notarangelo has received grants from the NIH and the March of Dimes; is an Associate Editor for the *Journal of Allergy and Clinical Immunology* and the *Journal of Clinical Immunology*; has consultant arrangements with Novimmune and Sigma-Tau; is employed by Children's Hospital Pediatric Associates; and has received royalties from UpToDate. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Ochs HD, Thrasher AJ. The Wiskott-Aldrich syndrome. J Allergy Clin Immunol 2006;117:725-39.
2. Dupuis-Girod S, Medioni J, Haddad E, Quartier P, Cavazzana-Calvo M, Le Deist F, et al. Autoimmunity in Wiskott-Aldrich syndrome: risk factors, clinical features, and outcome in a single-center cohort of 55 patients. Pediatrics 2003;111:e622-7.
3. Albert MH, Bittner TC, Nonoyama S, Notarangelo LD, Burns S, Imai K, et al. X-linked thrombocytopenia (XLT) due to WAS mutations: clinical characteristics, long-term outcome, and treatment options. Blood 2010;115:3231-8.
4. Li QZ, Zhou J, Wandstrat AE, Carr-Johnson F, Branch V, Karp DR, et al. Protein array autoantibody profiles for insights into systemic lupus erythematosus and incomplete lupus syndromes. Clin Exp Immunol 2007;147:60-70.
5. Catucci M, Castiello MC, Pala F, Bosticardo M, Villa A. Autoimmunity in Wiskott-Aldrich syndrome: an unsolved enigma. Front Immunol 2012;3:209.
6. Prete F, Catucci M, Labrada M, Gobessi S, Castiello MC, Bonomi E, et al. Wiskott-Aldrich syndrome protein-mediated actin dynamics control type-I interferon production in plasmacytoid dendritic cells. J Exp Med 2013;210:355-74.
7. Simon KL, Anderson SM, Garabedian EK, Moratto D, Sokolic RA, Candotti F. Molecular and phenotypic abnormalities of B lymphocytes in patients with Wiskott-Aldrich syndrome. J Allergy Clin Immunol 2014;133:896-9.e4.
8. Castiello MC, Bosticardo M, Pala F, Catucci M, Chamberlain N, van Zelm MC, et al. Wiskott-Aldrich Syndrome protein deficiency perturbs the homeostasis of B-cell compartment in humans. J Autoimmun 2014;50:42-50.
9. Du HQ, Zhang X, An YF, Ding Y, Zhao XD. Effects of Wiskott-Aldrich syndrome protein deficiency on IL-10-producing regulatory B cells in humans and mice. Scand J Immunol 2015;81:483-93.

http://dx.doi.org/10.1016/j.jaci.2015.08.010

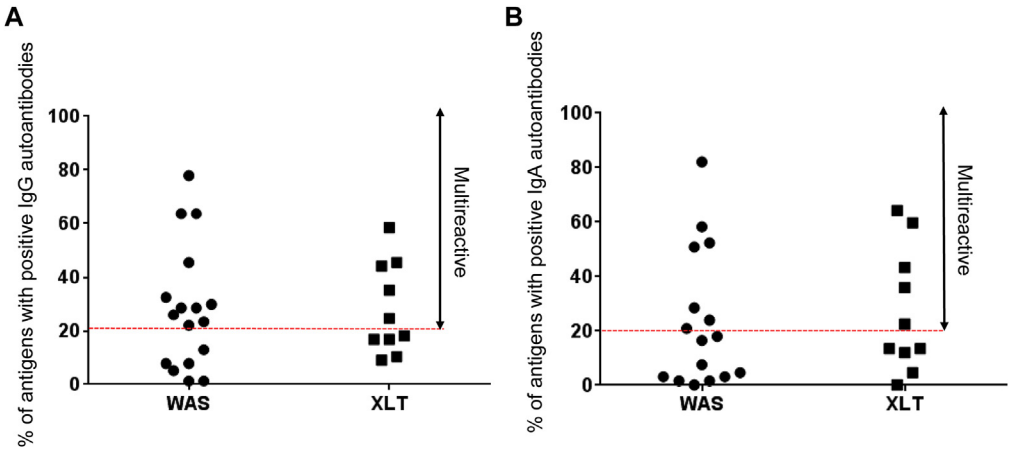

FIG E1. Percentage of self-antigens for which there were positive IgG **(A)** and IgA **(B)** autoantibody levels. Five hundred eighty autoantibodies were demonstrated in patients with WAS and those with XLT.

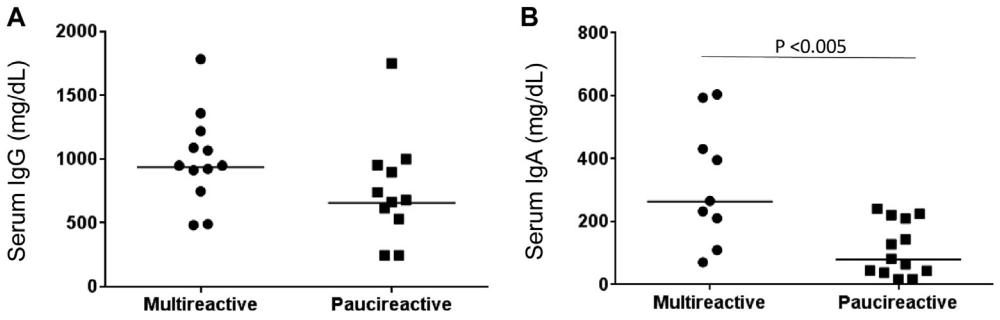

FIG E2. IgG **(A)** and IgA **(B)** serum levels in multireactive and paucireactive patients with WAS/XLT.

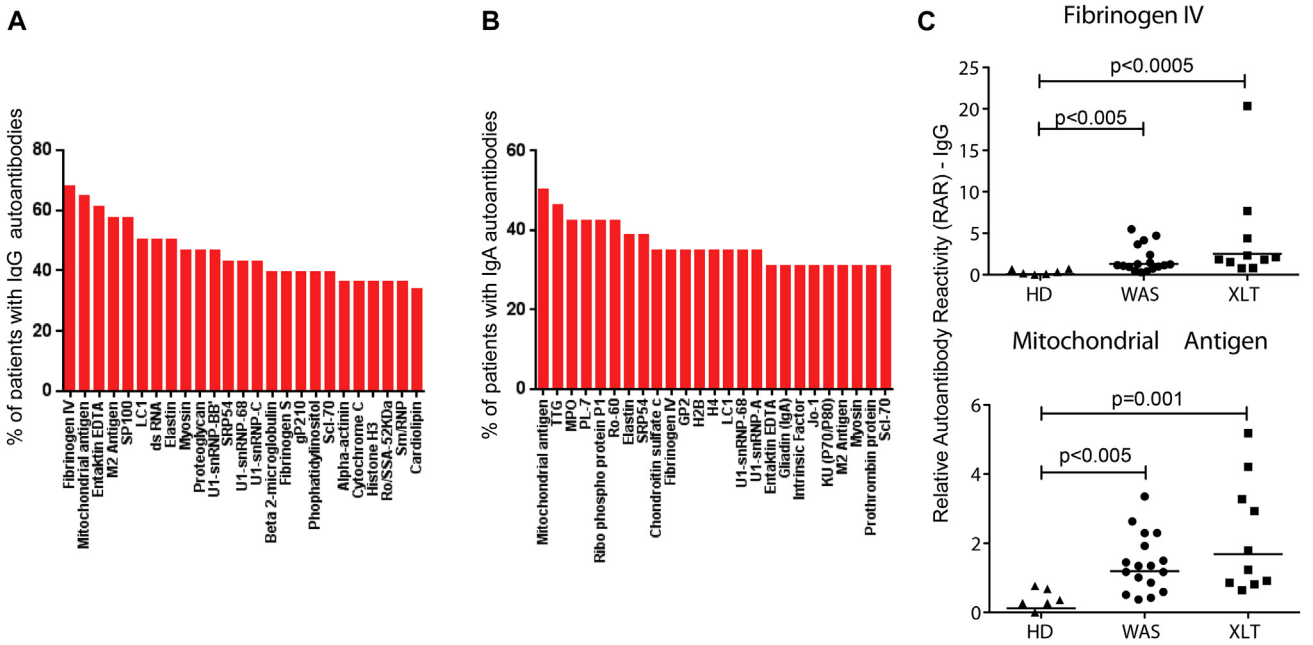

FIG E3. **A** and **B,** Frequency of positivity for the 25 most common autoantibodies. **C,** RAR values for 2 IgG autoantibodies (to fibrinogen IV and mitochondrial antigen) with significantly increased levels in patients with XLT and those with WAS compared with levels in healthy control subjects *(HD)*.