LABORATORY-BASED STUDY

Peritoneal autoantibody profiling identifies p53 as an autoantibody target in endometriosis

Sarah Harden, B.Sc.,[a,b,c] Tse Yeun Tan, M.B.B.S.,[d,e] Chee Wai Ku, M.D.,[d,e] Jieliang Zhou, M.Sc,[f] Qingfeng Chen, Ph.D.,[c] Jerry Kok Yen Chan, Ph.D.,[d,e] Jan Brosens, M.D., Ph.D.,[b,g,h] and Yie Hou Lee, Ph.D.[a,e,f]

[a] Critical Analytics for Manufacturing Precision Medicine, Singapore-MIT Alliance for Research and Technology, Singapore, Singapore; [b] Division of Biomedical Sciences, Clinical Science Research Laboratories, Warwick Medical School, University of Warwick, Coventry, United Kingdom; [c] Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore, Singapore; [d] Department of Reproductive Medicine, KKH, Singapore, Singapore; [e] OBGYN-Academic Clinical Program, Duke-NUS Medical School, Singapore, Singapore; [f] KK Research Centre, KK Women's and Children's Hospital, Singapore, Singapore; [g] Tommy's National Centre for Miscarriage Research, University Hospitals Coventry & Warwickshire, Coventry, United Kingdom; and [h] Centre for Early Life, Warwick Medical School, University of Warwick, Coventry, United Kingdom

Objective: To map the peritoneal autoantibody (AAb) landscape in women with endometriosis.
Design: Case-control laboratory study.
Setting: Academic medical and research units.
Patient(s): Women who presented with or without endometriosis.
Intervention(s): None.
Main Outcome Measure(s): Using native-conformation and citrullinated modified protein arrays, proteome-wide analysis of AAbs against 1,623 proteins were profiled in peritoneal fluids (PFs) of 25 women with endometriosis and 25 women without endometriosis.
Result(s): In women with endometriosis, the median number of AAbs detected was 4, including AAbs that targeted autoantigens involved in implantation, B-cell activation/development, and aberrant migration and mitogenicity. Forty-six percent of women with endometriosis have ≥ 5 peritoneal AAbs. Conversely, in women without endometriosis, the median number of detected AAbs was 1. Autoantibodies recognizing tumor suppressor protein p53 were the most commonly detected AAbs, being present in 35% of women with endometriosis, and p53 AAb was associated with a monocyte/macrophage-like PF cytokine signature. Further investigation of the global reactivity of AAbs against citrullinated PF antigens by peptidylarginine deiminase enzymes 1, 2, and 6 revealed anticitrullinated p53 as the only AAb target elevated and citrullinated by all 3 peptidylarginine deiminase isotypes. Furthermore, unsupervised hierarchical clustering and integrative pathway analysis revealed that 60% of women with endometriosis-associated infertility were positive for AAbs, which are involved in platelet-derived growth factor, transforming growth factor-β, RAC1/PAK1/p38/MMP2 signaling, LAT2/NTAL/LAB-mediated calcium mobilization, and integrin-mediated cell adhesion.
Conclusion(s): Together, our data identify peritoneal autoimmunity in a significant subset of women with endometriosis, with implications on infertility and disease pathophysiology. In these patients, p53 was identified as the most frequent PF AAb target, which was present in both the native and citrullinated forms. (Fertil Steril® 2023;■:■–■. ©2023 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, autoantibodies, autoimmunity, peritoneal, p53

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Correspondence: Yie Hou Lee, Ph.D., OBGYN-ACP, Singhealth-Duke-NUS Academic Medicine, 100 Bukit Timah Road, Singapore 229899; Singapore-MIT Alliance for Research and Technology, 1 CREATE Way, #04-13/14 Enterprise Wing, Singapore 138602 (E-mail: yiehou.lee@smart.mit.edu).

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Endometriosis is one of the most common gynecological disorders that affects up to 10% of women and one of the major causes of chronic pain and infertility. The disease is defined by the presence of endometrial-like implants found outside the uterus, most commonly on the peritoneum (1). The retrograde flow of shed endometrial tissues as the harbinger of foreign endometrial tissues and cells to extrauterine locations and subsequent implantation is commonly accepted as the principal

initiator of the disease (2). The appearance and ineffective clearance of these foreign and cell debris, including antigens in extrauterine locations during retrograde menstruation, potentially provoke autoimmune responses, immunologic tolerance, or rejection of the autograft with alloantigenic potential (3).

The plausibility of endometriosis being considered an autoimmune disease has been postulated, insofar that endometriosis meets most of the classification criteria of an autoimmune disease, and there is deregulation of the apoptotic process (4). Because endometriotic lesions originate from autologous cells containing self-antigens, it can be speculated that the abnormal exposure or presentation of these antigens facilitates an autoimmune response. This follows the discovery of immunoglobulin (Ig)G, IgM, and IgA autoantibodies (AAbs) directed against cell-derived antigens, such as phospholipids and histones (5). Antiendometrial and antiovarian AAbs against transferrin and alpha 2-HS glycoprotein were found in peritoneal fluids (PFs) of women with endometriosis (6, 7). Autoantibodies against endometrial and ovarian tissue in sera, vaginal, and cervical secretions in women with endometriosis suggest autoimmune dysregulation (6) and organ specificity (8). Furthermore, abnormalities in endometrial AAbs strongly suggest a role in endometriosis-associated infertility (EAI) (9). The association of endometriosis with autoimmune diseases and increased incidence of AAbs with endometriosis provide further support (3, 10–13). Interestingly, treatment with danazol or gonadotropin-releasing hormone analogs, which are commonly used as first- or second-line therapies for the treatment of endometriosis, suppressed the AAb levels (14, 15).

A defective peritoneal environment characterizes endometriosis, by which the PF rife with cytokines bathes the peritoneal cavity and surrounds endometriotic lesions (16). B-cell activating factor (BAFF or BLyS), a cytokine necessary for normal B-cell development, was up-regulated in endometriosis (17). Pathological analyses reported the presence of plasma cells (precursors of B cells), atypical B cells, and activated macrophages in endometriotic lesions (17). Intrinsic defects in peritoneal macrophages in endometriosis may also contribute to autoimmunity. Macrophages are important immune cells that maintain immune homeostasis via phagocytosis of foreign matter, apoptotic or necrotic cells, and are recruited to the peritoneum where they are prominently associated with endometriosis (18–20). Dysregulation in these immune cells promotes skewed tolerogenic peritoneal environments in endometriosis. Immunoglobulin G AAbs are approximately 150 kDa, and any interaction or exchange of AAbs in the PF with the circulation is limited due to the semipermeability of the peritoneum membrane (21). This, therefore, presents a unique microenvironment where locally produced AAbs are ineffectively cleared and retained in the peritoneal cavity and PF of women with endometriosis.

Although examples of autoimmune responses have previously been described, the comprehensive breadth of AAb reactivities in endometriosis remains undetermined. In this study, an integrated proteome-wide and bioinformatic analysis of >1,600 functional IgG native and citrullinated AAbs was performed in PFs of patients with endometriosis. We found that in close to half of patients with endometriosis, there are diverse autoreactivity and elevated AAb levels that target biological processes related to fertility, autoimmunity, and endometriosis pathophysiology. This is the first report that identified both the native and citrullinated forms of p53 as PF AAb targets in endometriosis. Citrullination is a posttranslational modification of arginine side chains into citrulline that produces non–self-neoepitopes, dramatically altering immunogenicity and driving further AAb production (22). Stratification by anticitrullinated p53 AAb positivity found a monocyte/macrophage PF cytokine signature. Together, these findings have important implications for stratification in endometriosis and the development of new therapeutic strategies against a subset of patients with endometriosis.

MATERIALS AND METHODS

Study Design and Patient Enrollment

Patients who underwent laparoscopic procedures at the KK Women's and Children's Hospital, Singapore, for various indications, such as suspected endometriosis, infertility, sterilization procedures, and/or pelvic pain, were recruited into the study. Women provided written informed consent for the collection of samples under Centralized Institutional Research Board approval (CIRB 2010-167-D).

The exclusion criteria include menstruating patients, postmenopausal patients, anovulatory patients, patients on any form of hormonal therapy for at least 3 months before laparoscopy, and other potentially confounding diseases, including diabetes, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and systemic sclerosis. Diagnostic laparoscopy was performed on all patients, with careful inspection of the uterus, fallopian tubes, ovaries, pouch of Douglas, and pelvic peritoneum by gynecologists subspecializing in reproductive endocrinology and infertility. The PF was prepared as previously described (8), in line with the Endometriosis Phenome and Biobanking Harmonization Project Standard Operating Procedures (23). The presence of endometriosis was systematically recorded and scored according to the revised American Fertility Society classification of endometriosis (24, 25) and classified as women with endometriosis (EM+, $N = 25$) or without endometriosis (EM−, $N = 25$). Women with infertility with endometriosis (EM+ EAI, $N = 15$) and without endometriosis (EM− EAI; $N = 13$) were extracted for the subsequent analysis. Patient characteristics are shown in Supplemental Table 1 (available online).

AAb Proteomics

Proteome-wide AAb profiling used the functional protein Immunome microarray platform (Sengenics, Singapore, Singapore), covering 1,623 wild-type antigens (Supplemental Table 2). This AAb protein array uses a compact, folded, biotinylated, domain with approximately 80 amino acid residues derived from the *Escherichia coli* biotin carboxyl carrier protein that preserves the structure and function of the embedded antigens, thus offering

exquisite selectivity and specificity of bounded AAbs (26, 27). The PF samples were diluted 1:200 in 2-mL dilution buffer (0.1% Triton X100 [v/v] and 0.1% bovine serum albumin [w/v] in phosphate-buffered saline) and applied to the array. The arrays were incubated in Quadriperm dishes (Greiner Bio One, Stonehouse, United Kingdom) and placed on a horizontal shaker at 50 rpm for 2 hours. After several washes, anti-human IgG was diluted 1:1,000 in assay buffer and Cy3-rabbit anti-human IgG (Dako Cytomation, Glostrup, Denmark) by incubation for 2 hours according to the manufacturer's recommendations. The array was washed and dried by centrifugation. All arrays using a microarray scanner (Axon 4200AL with GenePix Pro Software; Molecular Devices, San Jose, CA) and fluorescence of labeled IgG were detected according to the manufacturer's instructions. The interaction between microarray antigens and PF AAbs was detected as fluorescence of the bound fluorescently labeled IgG at the protein-specific position on the microarray. The intensity of fluorescence is proportional to the amount of AAb present in the PF. The local surrounding background intensity for each spot on the array was automatically subtracted from the relevant foreground intensity to give the net intensity for each spot; the median net intensity, also known as relative fluorescence units, was calculated from the quadruplicates of each antigen and was used for further analysis (Supplemental Fig. 1). This was applied across all spots and for cases and controls. All arrays passed quality control tests. The mean coefficient of variation percentage (CV%) of all protein replica spots across all samples was 8.12% (Supplemental Fig. 2A), and the CV% of Cy3-labeled biotinylated bovine serum albumin, which served as the positive control across slides and as a housekeeping probe for normalization of signal intensities across samples, was 8.08% (Supplemental Fig. 2B).

Citrullination Proteomic Analysis

The PF samples from 6 EM+ patients and 6 EM− women were diluted at 1:100 in dilution buffer, vortexed, and spun down. Subsequently, 30 μL of the sample was pipetted into 3 mL of wash buffer containing 0.2% v/v Tween 20 in phosphate-buffered saline and vortexed. Human peptidylarginine deiminase (PAD)1, PAD2, and PAD6 were chosen for further characterization because of their expression in the uterus and ovaries and importance to fertility (28–30) and are, therefore, relevant to endometriosis. Peptidylarginine deiminase 1, PAD2, and PAD6 were incubated on the protein array for the enzymatic conversion of arginine to citrulline. The protein array used adopted the correct folding of proteins (26), providing a more accurate reflection of in vivo functional citrullination. Briefly, each slide was rinsed in 3-mL wash buffer for 5 minutes. When the slides were rinsed completely, they were blocked in CT100plus blocking buffer for 1 hour. All slides were then washed 3 × 5 minutes in wash buffer at room temperature. The slides were then incubated with 3 mL of 1 μg/mL of human PAD1, PAD2, or PAD6, covered with aluminum foil, and incubated for 3 hours at 37°C at 50 rpm. The slides were washed for 3 × 5 minutes in wash buffer and then incubated with diluted PF samples on a horizontal incubator at 20°C for 2 hours. The citrullination protein array was then incubated with an anticitrulline antibody and fluorescently labeled detection antibody to detect the citrulline groups. Detection and hybridization signals were performed as previously described. The CV% of the intraprotein, intraslide, and interarray for all proteins and control probes of PAD1, PAD2, and PAD6 citrullinated protein arrays were 9.20%, 8.35%, and 6.63%, respectively, below the quality control limit of 15%.

Multiplex Immunoassay Analysis

The levels of 48 cytokines were measured in the PF fluid using a multiplex suspension bead immunoassay (BioRad, Hercules, CA; Supplemental Table 3) as previously described (16). Briefly, 10 μL of PF was mixed with 10 μL of primary antibody-conjugated magnetic beads on a 96 DropArray plate (Curiox Biosystems, Singapore) and rotated at 450 rpm on a plate shaker for 120 minutes at 25°C while protected from light. Subsequently, the plate was washed 3 times with wash buffer on the LT210 Washing Station (Curiox Biosystems) before adding 5 μL of the secondary antibody and rotating at 450 rpm for 30 minutes at 25°C protected from light. The plate was washed 3 times with wash buffer, and 10 μL of streptavidin-phycoerythrin was added and rotated at 450 rpm for 30 minutes at 25°C protected from light. The plate was washed 3 times with wash buffer, reading buffer was then added and transferred to a 96-conical-well microtiter plate, and the samples were read using the Bio-Plex Luminex 200 (BioRad). All samples were run in duplicates, and the mean was reported. Quantitation of the 48 cytokines in each sample was then determined by extrapolation to a 6- or 7-point standard curve using 5-parameter logistic regression modeling and concentration reported in pg/mL. Assay CV averaged <12%. Calibrations and validations were performed before runs and every month, respectively.

Data Analysis

For statistical analyses, parametric and nonparametric tests were used, with statistical significance set at $P<.05$. Hierarchical clustering was completed on the patients with infertility using the median distance and Manhattan clustering and portrayed as a heatmap. The concept and definition of penetrance (and of penetrance fold change [pFC]) have been adapted directly from the genetics field and seek to deal more appropriately with the analysis of markers, such as AAb markers that may be present at relatively low frequencies in a population and that would, therefore, not be found by *t* tests because of the high heterogeneity within the diseased cohort. Therefore, in our analysis pipeline, penetrance referred to the proportion of individuals with an AAb against a particular antigen. In the pFC method, antigens in the dataset that had a net intensity value in the EM+ cohort that was greater than 2 standard deviations of the mean net intensity measurements made for each antigen in the control cohort (EM−) were first identified; we then filtered that antigen list on the basis of a minimum frequency for each individual in the EM+ cohort on the basis of a cutoff of 4%, and a fold change for each surviving antigen was calculated, on the

basis of the comparison of the mean net intensity of the antigen in EM+ individuals where it is present, relative to the net intensity of that antigen in the control cohort. Equations on pFC are shown in Supplemental Figure 3. To understand the change and ultimately the importance in interpretation of AAbs associated with endometriosis, a fold change of ≥ 2 was used. A fold change of ≥ 2 was a better eliminator of potential spurious background signals that could have arrived from concentration-driven, nonselective binding because there were fewer AAbs left after making a fold change cutoff from ≥ 1.5 (Supplemental Table 4). As the fold change level increased to that of ≥ 3, the number of AAbs significantly decreased, which suggested too stringent a cutoff. In addition, in determining significant fold change cutoff, defined using AAbs levels of >2 SD of the control population, a fold change ≥ 2, but not 1.5, fit into the criteria.

Pathway Enrichment Bioinformatic Analysis

Functional enrichment data were obtained from ToppGene Suite on the basis of Gene Ontology, pathways, and disease, using the ToppFun tool (31). *P* values were calculated using the probability density function and false discovery rate Benjamini and Hochberg corrected, with a corrected *P* value ($q < 0.05$). The OpenTargets Platform was used to find proteins associated with autoimmunity and endometriosis (32). Here, the overall association scores between a target and disease were calculated using data from multiple sources and adjusted depending on data source and type. A value of 1 represented the most associated. These proteins were cross-referenced to the proteins identified as AAbs to highlight proteins known to be involved in autoimmunity, endometriosis, or both.

RESULTS

Diversity of Peritoneal AAbs in a Subset of Patients with Endometriosis

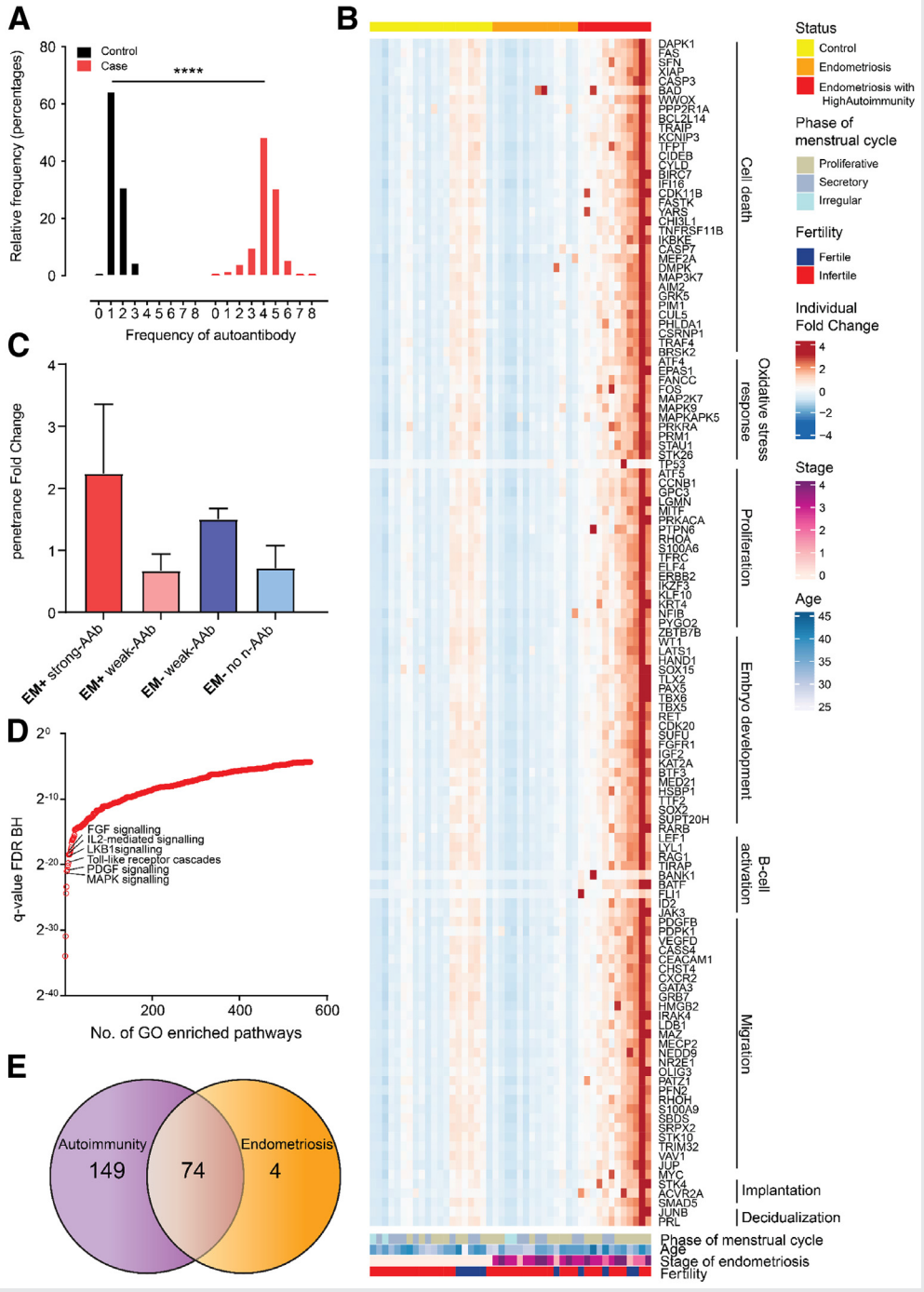
Of the analyzed 1,623 IgG AAbs, 351 discrete AAbs were putatively considered as significant (pFC_{Case}, ≥ 2; penetrance $Frequency_{Case}$, $\geq 20\%$; Supplemental Table 4). Previously identified PF and endometrial tissue AAbs in endometriosis, antihistone H1.2, and anti-AHSG AAbs were validated (pFC, 3.59 and 3.24; penetrance $Frequency_{Case}$, 20% and 24%, respectively; Supplemental Fig. 4A and B) (9, 33). In EM+ patients, 84.1% of PF AAbs were found at frequencies of 4–6 (Fig. 1A). This contrasted with EM− controls, whereby 95.1% of PF AAbs were presented at a low frequency of 1 and 2 (Kruskal-Wallis test corrected with Dunn's test for multiple comparisons, $P < .0001$). By hierarchical clustering, a cluster of EM+ cases (46% or 12/25) with strong autoimmune profiles (≥ 5 significant AAbs per patient; mean pFC, 2.24 vs. 0.67 for the rest of EM+; $P < .0001$ for both the mean or median fold change and number of AAbs with a pFC of ≥ 2) were observed (Fig. 1B and C), although the cluster was not associated with menstrual phase, age, or pregnancies. Comparing the proliferative and secretory phases in EM−, there were 21 significantly different AAbs (Supplemental Table 5). In EM+, there were 6 significantly different AAbs, suggesting the retention of AAbs in endometriosis across the menstrual cycle. None of the high-frequency AAbs were affected by the menstrual cycle phases. Many of the EM+ AAbs included markers of fertility such as decidualization (PRL) and implantation (ACVR2A and SMAD5), autoimmunity such as B-cell activation and development (BANK1 and FLI1), endometriosis pathophysiology such as migration (TIMP3 and MMP24), and mitogenicity (PDGFB, PDGFRL, FGFR1, FGFR2, IGF2, and VEGF-D) (Fig. 1B). No evidence of autoimmunity against cytokines or chemokines was observed (Supplemental Fig. 4C to J). Pathway enrichment indicated that the AAbs were found to elicit MAPK ($q = 4.68 \times 10^{-7}$), platelet-derived growth factor (PDGF) ($q = 5.51 \times 10^{-7}$), LKB1 ($q = 2.74 \times 10^{-6}$), FGF ($q = 4.81 \times 10^{-6}$), interleukin (IL)-2-mediated signaling ($q = 3.05 \times 10^{-6}$), and Toll-receptor signaling ($q = 1.13 \times 10^{-6}$) (Supplemental Table 5 and Fig. 1D). The 351 AAbs were cross-referenced to potential targets under "Autoimmunity" and "Endometriosis" disease categories using the OpenTargets Platform. A total of 149 AAbs were found to be associated with "Autoimmunity," 4 AAbs were found to be associated with "Endometriosis," and 74 AAbs overlapped with both "Endometriosis" and "Autoimmunity" (Supplemental Tables 6 and 7 and Fig. 1E).

Elevated Levels of Antinative and Citrullinated p53 Antibodies in Endometriosis Is Associated with Monocyte-Associated Cytokine Profile

The most frequently occurring EM+ AAb was p53, which was detected in 35% of EM+ patients and 58% in the high-autoimmunity EM+ cases (Fig. 2A). The anti-p53 AAb level was significantly elevated in EM+ patients compared with that in EM− patients (mean pFC_{case}, 6.46, vs. mean $pFC_{control}$, 0) and was not associated with American Society for Reproductive Medicine stage, age, or menstrual phase. The PF of EM− patients did not show positivity for anti-p53. Therefore, anti-p53 AAb was used to stratify EM+ patients into $p53^{high}$ ($pFC_{p53} > 2.0$) and $p53^{low}$ ($pFC_{p53} < 2.0$) for further investigation of whether the presence of anti-p53 AAb influenced the peritoneal inflammatory environment and the frequency of citrullinated p53.

Because p53 provokes inflammatory responses by modulating immunologic changes (34, 35), we hypothesized that the presence of anti-p53 AAbs altered the PF cytokine milieu. To test this, we performed multiplex suspension bead immunoassay of PF cytokines on EM+ $p53^{high}$ samples compared with EM+ $p53^{low}$ and EM− samples. Forty PF cytokines were detected (Supplemental Table 3). A striking monocyte/macrophage-related chemokine signature comprising of significantly elevated levels of IL-6, interferon-γ (IFNγ), monocyte chemoattractant protein (MCP)1, and MCP3 and reduced monokine induced by IFNγ levels distinctly marked $p53^{high}$ samples (Fig. 2B). Monocyte chemoattractant protein 1 and MCP3 are monocyte/macrophage chemoattractants (36). The secretion of monokine induced by IFNγ by predominantly monocytes/macrophages is induced by IFNγ and mediated by the Janus kinase-signal transducer and activator of transcription signaling pathway. The IL-6 levels were significantly higher in $p53^{high}$ than those in EM−.

FIGURE 1



Women with endometriosis had diverse peritoneal autoantibodies (AAbs). (**A**) Distribution histogram of the frequency of positive AAbs with a penetrance fold change of ≥2, showing that more women with endometriosis (EM+) ($n = 25$) possessed higher frequencies of AAbs, median of 4, than women without endometriosis (EM− controls), with a median of 1 AAb ($n = 25$). (**B**) Heatmap of 351 AAb levels in women with ($n = 25$) and without ($n = 25$) endometriosis. The AAb levels were Z score normalized against the control population mean and standard deviation, with Z scores of >2 corresponding to positive AAb levels. There were 3 major AAbs clusters: control; endometriosis; and endometriosis with high autoimmunity. Endometriosis with high autoimmunity, ≥5 AAbs at a fold change of ≥2; endometriosis with weak autoimmunity, patients with endometriosis who did not meet the criteria. When comparing the mean or median fold change in all AAbs per patient, weak vs. high autoimmunity had a P value of <.0001. Hierarchical clustering was completed using the median distance and Manhattan clustering. (**C**) Bar graphs of EM+ cases and EM− controls with strong (≥5 significant AAbs per patient) and weak (<5 significant AAbs per patient) autoimmune profiles. (**D**) Pathway enrichment q values (false discovery rate Benjamini-Hochberg) indicated that positive AAbs in endometriosis were found to elicit pathways involved in MAPK ($q = 4.68 \times 10^{-7}$), PDGF ($q = 5.51 \times 10^{-7}$), Toll-receptor signaling cascade ($q = 1.13 \times 10^{-6}$), LKB1 ($q = 2.74 \times 10^{-6}$), interleukin (IL)-2-mediated signaling ($q = 3.05 \times 10^{-6}$), and FGF ($q = 4.81 \times 10^{-6}$) as top ranked pathways. (**E**) Number of the 351 AAbs associated with autoimmunity, endometriosis, or both, as determined by the OpenTargets Platform.

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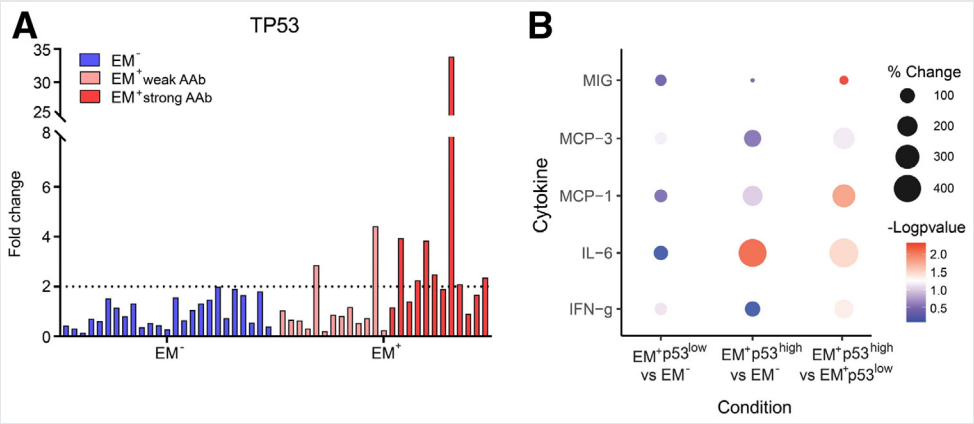
FIGURE 2



Predominance of anti-p53 autoantibody in endometriosis. (**A**) Bar graph of anti-p53 autoantibody fold change, showing that the autoantibody levels were elevated in women with endometriosis (EM+). (**B**) Dot plot of chemokines indicative of changes in cytokine milieu in patients with endometriosis with high p53 autoimmunity. The size of the dots denotes the percentage change in cytokine secretion, and the color is indicative of the significance. A -log *P* value of >1.3 was the same as *P*<.05.

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For the study of citrullinated targets, we pooled PF samples from EM+ patients on the basis of their levels of anti-p53 AAb into 2 anti-p53 AAb groups ($p53^{high}$ and $p53^{low}$) and compared them with those from EM− women (Fig. 3A). Embedded antigens in the protein array were then citrullinated in vitro with PAD isoforms 1, 2, and 6 and probed using anticitrullinated antibodies in the PF samples. Known citrullinated proteins keratins (KRT15 and KRT19), vimentin (VIM), and aldolase (ALDOA) were observed, thereby validating the assay (Supplemental Fig. 5A). The anti-p53 AAb groups had different citrullination patterns depending on whether they were incubated with PAD1, PAD2, or PAD6, with PAD1 generating the most autoantigens (Fig. 3B and Supplemental Fig. 5B). In the $p53^{high}$ group 72 citrullinated AAbs overlapped in AAbs generated by the 3 PADs and in the $p53^{low}$ group, only 1 overlapped. Overall, the list of anticitrullinated AAbs overlapped minimally with that of noncitrullinated AAbs. Interesting, citrullinated p53 was the only target among the anticitrullinated AAbs that were common to PAD1, PAD2, and PAD6 and noncitrullinated AAbs (Fig. 3C). It was approximately 1.6 times higher in the $p53^{high}$ group than in the $p53^{low}$ EM+ and EM− groups (Fig. 3D).

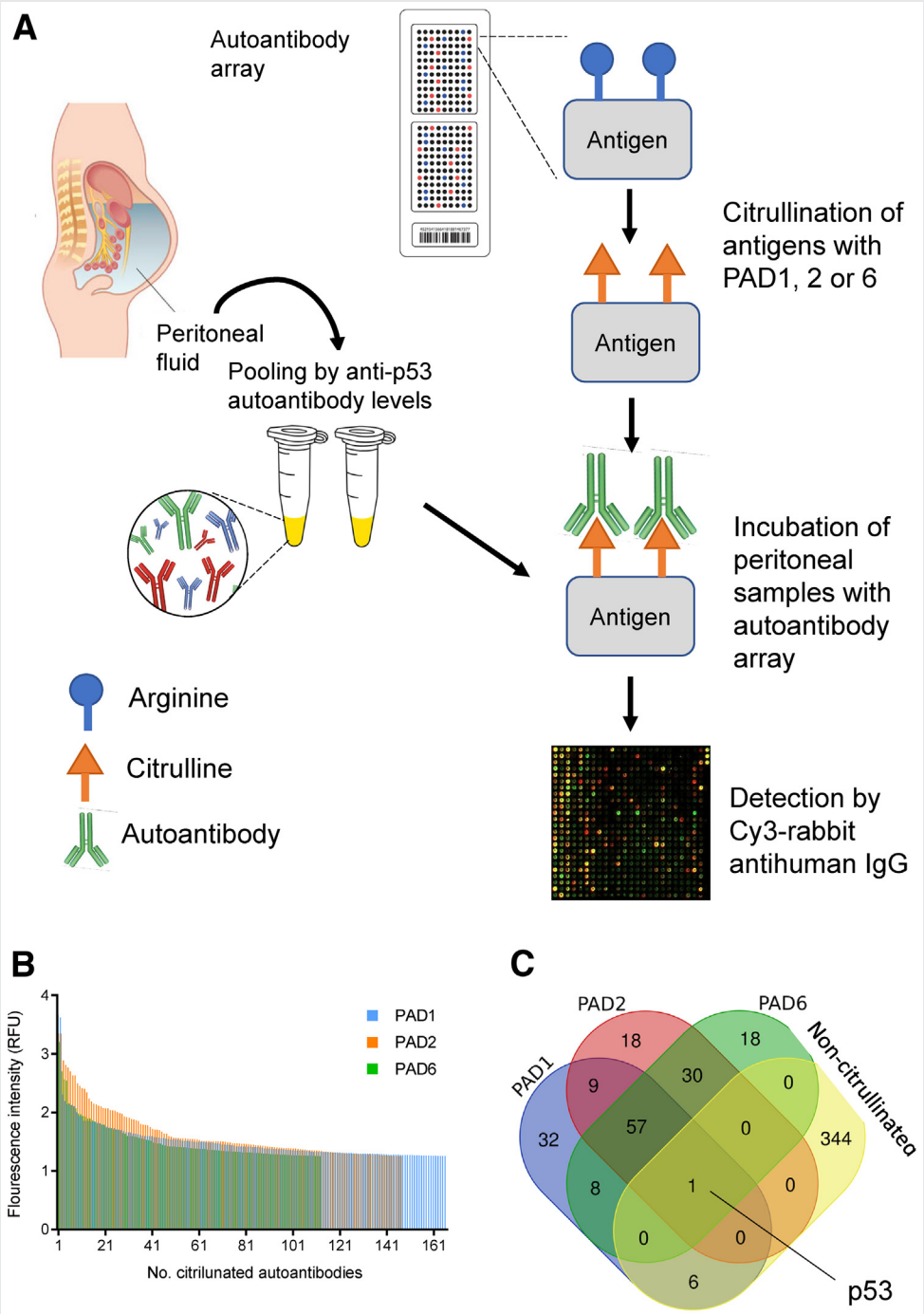
Discovery of Novel Peritoneal AAbs and p53 in EAI

Autoimmunity in EAI potentially works through different putative mechanisms or etiology (37). Additional analysis on 15 infertile EM+ patients and 13 infertile EM− age- and ethnicity-matched healthy controls selected from the aforementioned study was performed. The levels of 109 AAbs were elevated in EAI EM+ subjects (pFC, ≥2; penetrance $Frequency_{Case}$, ≥20%). Moreover, 60% (or 9/15) of EAI cases testing positive for multiple AAbs (≥2 AAbs with elevated levels) were observed (Fig. 4A, patients denoted with purple status). No AAb level was elevated in infertile EM− individuals. The most prevalent AAb in EAI patients was TAF9 (frequency, 33.3%). Anti-p53 AAb was prevalent at 27% frequency in EAI (Fig. 4B). Twenty-seven percent of them were also positive for BAD, SEPTIN4, C1D, NFE2L2, MAPK1, ELF1, CSNK1G1, COQ8A, and ZNRD1 (Supplemental Table 8). Interestingly, anti–ceramide transport protein (also known as COL4A3BP) AAbs levels were elevated in 20% of cases, consistent with our earlier sphingolipidomic analysis of aberrant ceramide metabolism in EAI (38). When examining AAbs that were common between endometriosis and autoimmunity, analysis of 24 proteins (22%), including p53, showed that these were found to be associated with both endometriosis and autoimmunity (Supplemental Table 9). Integrative pathway analysis demonstrated enrichment in AAbs involved in PDGF signaling ($q = 7.62 \times 10^{-5}$), transforming growth factor-β signaling ($q = 0.0007$), LAT2/NTAL/LAB-mediated calcium mobilization ($q = 0.0007$), integrin-mediated cell adhesion ($q = 0.0009$), and the RAC1/PAK1/p38/MMP2 signaling axis ($q = 0.0009$) (Supplemental Table 10). The enrichment of NTAL/LAB/LAT2 pathway, found in activated B cells and monocytes (39), further suggests the implication of B-cell mediated autoimmunity in EAI. Integrin-mediated and transforming growth factor-β pathways have been implicated in fertility and endometriosis (40–42).

DISCUSSION

Peripheral and endometrial AAbs that are associated with endometriosis have been reported (5, 43). In this study, we investigated 1,623 immunoreactive antigens against extracellular IgG AAbs found in PF, confirming the detection of AHSG and histones reported in earlier studies and reporting herein on novel AAbs. Close to half of EM+ patients (46%) have a strong autoimmune profile with ≥5 AAbs detected per patient. The female preponderance to an increased likelihood of autoimmunity and endometriosis-associated

FIGURE 3



Identification of citrullinated autoantibodies in endometriosis. (**A**) Schematic of citrullinated autoantibody protein array generation for profiling. Peritoneal fluids from patients with endometriosis (EM+) on the basis of their levels of anti-p53 autoantibody levels into 2 anti-p53 autoantibody groups: $p53^{high}$ and $p53^{low}$. (**B**) Different citrullination patterns were obtained depending on whether they were incubated with peptidylarginine deiminase (PAD)1, PAD2, or PAD6, with PAD1 generating the most reactive autoantigens. (**C**) In the $p53^{high}$ group, 72 citrullinated autoantibodies overlapped in autoantibodies generated by the 3 PADs, and in the $p53^{low}$ group, only 1 overlapped. Overall, the list of anticitrullinated autoantibodies overlapped minimally with that of noncitrullinated autoantibodies. Citrullinated p53 was the only target among the anticitrullinated autoantibodies that were common to PAD1, PAD2, and PAD6 and that overlapped with noncitrullinated autoantibodies. IgG = immunoglobulin G.

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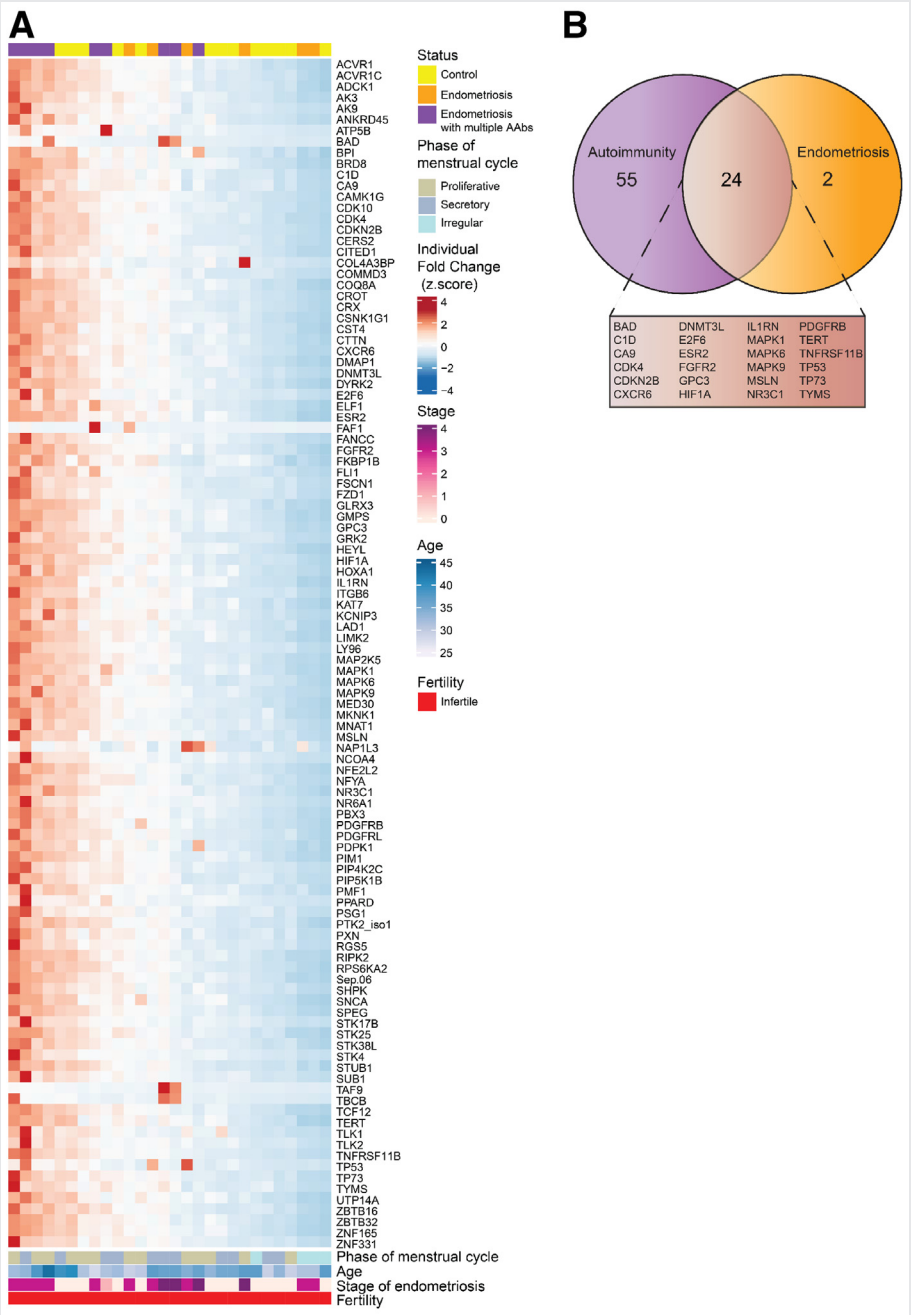
FIGURE 4



Peritoneal autoantibodies (AAbs) in endometriosis-associated infertility. (**A**) Unsupervised clustering heatmap of 109 AAb levels in women with (n = 15) and without (n = 13) endometriosis. The AAb levels were Z score normalized against the control population mean and standard deviation, with Z scores of >2 corresponding to positive AAb levels. Hierarchical clustering was completed using the median distance and Manhattan clustering. (**B**) Number of the 109 AAbs associated with autoimmunity, endometriosis, or both, as determined by the OpenTargets Platform.

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autoimmunity may be explained by estrogen and retrograde menstruation. Activation-induced deaminase deaminates cytosines at immunoglobulin loci, initiating a cascade of events that lead to somatic hypermutation and class switch recombination, turning IgG AAbs pathogenic. Activation-induced deaminase has been reported to be estrogen-induced (44), and in ovarian tissues where the estrogen levels are high, deleterious insertions of point mutations or the resolution of double-strand breaks potentially accumulates over time, generating pathogenic AAbs (44). The presence of live endometrial cells and cellular debris in the peritoneal cavity as a result of retrograde menstruation and their defective

clearance in endometriosis presents a favorable environment that results in abnormal exposure of autologous antigens to the immune system and, therefore, triggers the initiation of an autoimmune response in the peritoneal environment (45, 46). In this study, we showed diverse peritoneal autoimmune responses that varied from patient to patient with endometriosis. One possibility is that autoreactive lymphocytes expand polyclonally, have different antigen receptors on their surface, and, thereby, recognize different targets. Another possibility is the initial autoimmune response in an inflammatory peritoneal environment expands to include self-proteins through linked recognition and intermolecular epitope spreading.

The tumor suppressor p53, encoded by the *TP53* gene, is a deoxyribonucleic acid motif binding transcription factor that governs core cellular programs to ensure cell and tissue homeostasis, including arresting cell cycle progression and apoptotic response to cellular stress (47). As evidenced in Proteinatlas, p53 is barely detectable in endometrial stromal or epithelial cells (48, 49) but is highly expressed in monocytes and B and T cells (50). Although p53 expression in endometriosis has been controversial (51–53), our data are consistent with anti-p53 AAbs being restricted to patients with mutated forms of p53. Different patterns of TP53 mutations have been reported in endometriosis, including missense mutations, deletion of the *TP53* locus, and loss of heterozygosity that can contribute to or trigger an immune reaction by causing self-immunization of non–wild type p53 (54–56). There is a strong correlation between the frequency of anti-p53 AAbs and that of p53 mutations in certain types of cancer, suggesting that p53 mutations are associated with the generation of these AAbs (54). Studies in mice revealed the close association of p53 deficiency with the development of autoimmune and inflammatory diseases (57, 58). In particular, monocytes/macrophages deficient in p53 inefficiently clear apoptotic and necrotic cells, and the failure to clear dying cells can lead to accumulation of autoantigens that promote further generation of autoimmunity and chronic inflammation (57, 59). Citrullinated p53 has previously been reported (60, 61) but not in endometriosis. This study showed that women with endometriosis have citrullinated anti-p53 AAbs. The incubation of PF with citrullinated antigens converted by PAD1, PAD2, and PAD6 identified a diversity of anticitrulline AAbs, including p53, suggesting that PADs found within the peritoneal environment are responsible for citrullinating proteins. Our finding of anticitrullinated p53 AAbs further renders p53 as a potential target of pathological autoimmunity in endometriosis.

There are important biomedical and clinical ramifications of this study. Developing and/or identifying relevant animal models would be instrumental in allowing the investigation of various aspects relevant to the role of PF AAbs in general, and of anti-p53 AAbs in endometriosis and EAI, and for the testing of plausible treatment options (62). Autoantibodies recognizing p53 were the most frequently detected in 35% of EM+ patients. If anti-p53 AAbs and associated signaling pathways represent a set of novel underlying pathogenic mechanisms in endometriosis, the prediction is that anti-p53 AAb-positive patients may benefit from different treatment strategies. Diagnostic laparoscopy visually captures the "static" snapshot of the peritoneal cavity, which is insufficient given the growth-and-regress cycles of estrogen-driven endometriotic lesions (63), and potentially misses out on regressed lesions that would only recur in subsequent cycles. Autoantibodies are stable over long periods (64, 65), even in the presence of low corresponding antigen levels (66). Anti-p53 AAb can potentially be used to identify women with past episodes of endometriosis but escape current diagnosis via laparoscopy. A correlational study of serum p53 AAb with PF p53 AAb would provide information on whether serum p53 AAb can be used as a predictive non-invasive marker to identify subsets of endometriosis patients who would benefit from targeted therapies.

A key strength of this study is the use of a protein array that immobilizes the correct 3-dimensional folding of full-length proteins (26). This has several important advantages on the accuracy of our results, including the following: maximizing interactions between AAbs and immobilized proteins while greatly minimizing the false positives that otherwise arise from nonspecific binding of AAbs; a more accurate reflection of in vivo functional citrullination; and the anti-p53 AAbs being able to target both wild-type and mutant p53 that have been reported in the PF of women with endometriosis (54–56). This study is the largest to date, which identified local autoimmunity in the peritoneal cavity in EM+ patients and EM− controls. Validation of previously identified AAbs confirmed the identification of novel bona fide AAb. Finally, and perhaps most importantly, the advantage is the identification of AAbs in the PF, considered the environment that is most proximal to lesions and, thereby, capturing key cognate antigen-AAb interactions in endometriosis.

Several studies of endometriosis, including this study, have important limitations. First, the heterogeneous nature of the disease, in clinical presentation, underlying pathogenesis and genetics, and demographics, coupled with a single timepoint for sample collection, may have contributed to the heterogeneous AAb profiles that potentially confounded interpretation. Second, Although the employed AAb array covered >1,600 proteins, autoimmunity that may be elicited by other potential autoantigens, such as lipids, carbohydrates, deoxyribonucleic acid, ribonucleic acid, and signaling and hydrophobic proteins, were not captured. Finally, our analysis was limited to a relatively small study population, and results should be interpreted with caution unless confirmed in future studies with greater granularity on specific clinical and/or demographic features.

In summary, this study provides an expansive peritoneal AAb landscape in patients with endometriosis and identified p53 as a high-frequency AAb target that defined its association in autoimmunity. These results suggest the causal inference of p53 and previously underappreciated pathways that are linked to the autoimmunologic etiology of endometriosis, with implications on novel therapeutic paradigms centered on modulating these pathways and potentially immune cells to explore endometriosis immunotherapies.

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