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Pulmonary Autoimmunity as a Feature of Autoimmune Polyendocrine Syndrome Type 1 and Identification of KCNRG as a Bronchial Autoantigen

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Short title: Pulmonary Autoimmunity in APS-1 and Identification of KCNRG as Autoantigen

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Abstract

Patients with autoimmune polyendocrine syndrome type 1 (APS-1) suffer from multiple organ-specific autoimmunity with autoantibodies against target tissue-specific autoantigens. Endocrine and also non-endocrine organs such as skin, hair follicles and liver are targeted by the immune system. Despite sporadic observations of pulmonary symptoms among APS-1 patients, an autoimmune mechanism for pulmonary involvement has not been elucidated. We report here on a subset of APS-1 patients with respiratory symptoms. Eight patients with pulmonary involvement were identified. Severe airway obstruction was found in four patients leading to death in two. Immunoscreening of a cDNA library using serum samples from a patient with APS-1 and obstructive respiratory symptoms identified a putative potassium channel regulator (KCNRG) as a pulmonary autoantigen. Reactivity to recombinant KCNRG was assessed in 110 APS-1 patients using immunoprecipitation. Autoantibodies to KCNRG were present in seven of the eight patients with respiratory symptoms, but in only one of nine APS-1 patients without respiratory symptoms. Expression of KCNRG messenger RNA and protein was found to be predominantly restricted to the epithelial cells of terminal bronchioles. Autoantibodies to KCNRG, a protein mainly expressed in bronchial epithelium, are strongly associated with pulmonary involvement in APS-1. This finding will facilitate the characterisation and diagnosis of the pulmonary manifestations of APS-1.

Introduction

Autoimmune Polyendocrine Syndrome Type 1 (APS-1), also known as Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED, OMIM 240300), is a rare disorder caused by mutations in the Autoimmune Regulator (AIRE) gene¹. Patients with APS-1 progressively develop multiple organ-specific autoimmunity of endocrine and non-endocrine tissues. Loss of function of the Aire protein results in decreased expression of self-antigens in medullary thymic epithelial cells and in failure to establish central tolerance to a range of different autoantigens². Multiple autoantibodies directed against specific intracellular autoantigens are found. Well defined autoantigens in APS-1 include 21-hydroxylase in the adrenal cortex, tryptophan hydroxylase in intestinal serotonin-producing cells and NACHT, leucine rich repeat and PYD containing 5 (NALP5) in parathyroid glands³⁻⁵. Detection of autoantibodies can help in the diagnosis of a disease component or predict its future development^{5,6}. Hypoparathyroidism and Addison's disease are the most frequent disease components, and more than 20 different autoimmune manifestations have been identified in APS-1^{1,6,7}. Major histocompatibility complex (MHC) and non MHC allelic variation are believed to influence disease expression^{8,9}. Identification of tissue-specific autoantigens in APS-1 provides useful diagnostic markers and increases our understanding of the variable expression of disease in individuals⁶.

Although pulmonary disease has been sporadically observed in APS-1 patients, an immune-mediated mechanism has not been established^{10,11}. We describe APS-1 patients with autoimmune pulmonary disease, and a potassium channel regulating protein preferentially expressed in the epithelial cells of terminal bronchioles (KCNRG) as the putative target antigen.

Results

Identification of Airway Inflammation as a Component of APS-1

Severe respiratory symptoms were seen in four of 110 APS-1 patients described below (patients 1—4, **Table 1**) Five additional cases with respiratory involvement and/or KCNRG autoantibodies were subsequently identified after recruitment to the study (patients 5-9, **Table 1**).

Patient 1 developed asthma-like respiratory symptoms aged 5 years. At age 10 the symptoms worsened, and were poorly controlled by inhaled and oral glucocorticoids. By 11, APS-1 was diagnosed. Lung function tests showed obstruction with reduced forced vital capacity (FVC), forced expiratory volume (FEV₁) and forced expiratory flow (FEF 25-75%) at 73%, 48% and 26% of predicted. Skin prick tests were negative for all allergens tested. Chest CT scan showed bronchiectasis with peribronchial ground-glass opacities (**Figure 1A**). Treatment with prednisolone (2 mg/kg/d) markedly improved the respiratory symptoms, but relapses were severe on dose reduction. Lung biopsy showed inflammation with peribronchiolar lymphoid infiltrate in the small bronchioles (**Figure 1C and D**). Azathioprine was started at 14 years of age with little steroid-sparing effect. Mycophenolate mofetil (750 mg twice daily) was commenced at age 15.5 years, with good effect and prednisolone was decreased to 0.14 mg/kg/d. Respiratory symptoms and CT scan appearances improved and improvement was maintained at evaluation 2.5 years later (**Figure 1B**).

Patient 2 was diagnosed with APS-1 in early childhood, but died at the age of 18 of cor pulmonale and respiratory failure. Autopsy was not performed. From the age of 5 he had lower respiratory tract infections at least 2-3 times a year. Chest X-ray and CT scan revealed bilateral bronchiectasis from the age of 10 years. Lung function tests showed reduced FEV₁ (< 40%) and FVC at 50-60% of predicted. His respiratory symptoms deteriorated with exercise intolerance, shortness of breath and growth failure. Exacerbations were poorly controlled by cycles of antibiotic and glucocorticoid therapy. Chronic colonization with *Burkholderia cepacia* developed. By age 14 years he was

oxygen dependent with FEV₁ and FVC at 14% and 13%, respectively of expected. Other causes were excluded by extensive investigations including sweat test, nasal mucosal brush biopsy and genetic analysis for cystic fibrosis.

Patient 3, currently 19 years old, was diagnosed with hepatitis due to APS-1 aged 9 months. Dyspnoea in early childhood was initially diagnosed as asthma. By age 10 years, bronchiolitis obliterans organizing pneumonia had developed, with bronchiectasis on the CT scan verified by lung biopsy (**Figure 1E and F**). He suffered recurrent lower respiratory tract infections. He is currently oxygen-dependent with an FVC and FEV₁ at 31% and 18%, respectively of predicted.

Patient 4 presented with chronic cough in childhood. APS-1 was diagnosed at 9 years of age. By 20, he had established bronchiectasis on chest X-ray and CT scan. Spirometry showed airway obstruction. The patient had frequent episodes of “infectious bronchitis” and gradually deteriorated. At age 34 years, he was admitted to intensive care because of hypoxemic pneumonia. Lung biopsy showed a severe peribronchiolar infiltrate (**Figure 1G and H**). He died of chronic respiratory failure at the age of 37.

Immunoscreening of a cDNA Library and Autoantibody Assay on KCNRG

By immunoscreening a bovine cDNA library with serum from patient 6 with obstructive pulmonary disease and hypoparathyroidism, we found three independent clones encoding KCNRG (GenBank accession number AY190923). KCNRG-specific autoantibodies were subsequently sought in sera from 105 APS-1 unselected patients independent of the presence of respiratory symptoms. Four of these 105 sera (patients 6 to 9 in **Table 1** - including patient 6 used for immunoscreening) were positive. None of 252 control sera were positive (**Figure 2A**). These findings led us to test for immunoreactivity to KCNRG in sera from APS-1 patients selected for the presence of severe pulmonary disease (patient 15, **Table 1**). Four of these five patients (patient 14, **Table 1**) had high titer antibody. In total 8 of 110 (7.2 %) APS-1 patients investigated, displayed the KCNRG autoantibodies.

Expression Analysis of KCNRG Messenger RNA and Protein

Microarray expression databases such as GNF SymAtlas and GeneNote, state that tissue expression of KCNRG is almost ubiquitous^{17,18}. Nevertheless, we investigated the tissue expression of KCNRG by Northern blotting and quantitative real-time PCR. Northern blot (**Supplementary Figure 1**) demonstrated that expression of KCNRG was actually restricted to the lungs. Quantitative PCR analysis (**Figure 2B**) showed that, mRNA expression of KCNRG was predominantly restricted to the lungs. However, it also revealed that KCNRG mRNA was expressed to a low extent in the pancreas and prostate. Nevertheless, expression in other tissues was at much lower levels than in lungs. Immunohistochemistry on bovine lung using a rabbit polyclonal antiserum developed against KCNRG, specifically stained epithelial cells of the terminal bronchioles (**Figure 3B**). To further exclude the ubiquitous expression of the KCNRG at protein level, we used the antiserum directed against KCNRG and stained a human multi-tissue array. The results were in line with the results for mRNA expression experiments (**Supplementary Figure 6**).

Immunostaining of Lung Tissue with APS-1 Sera

Immunofluorescence of bovine lung with anti-KCNRG positive sera from APS-1 patients, showed specific staining of the epithelial cells of terminal bronchioles with two of the three sera used (**Figure 3 E-F**). The staining pattern of patient sera was identical to that of the KCNRG-antiserum. No staining was seen with control sera from anti-KCNRG negative APS-1 patients (**Figure 3 H-I**) or healthy blood donors (**Figure 3 J-K**).

The specificity of APS-1 patient autoantibodies for KCNRG, was confirmed by preabsorption studies. In these experiments, preabsorption of the patient serum samples with recombinant

KCNRG abolished the staining of the terminal bronchioles (Supplementary Figure 5 B and E). In contrast, preabsorption of the sera with equal amount of Luciferase did not reduce specific staining of the terminal bronchioles (Supplementary Figure 5C and F).

Discussion

We present evidence showing that pulmonary autoimmunity is a component of APS-1 and have identified the KCNRG protein as a target autoantigen. Strong serum reactivity against KCNRG was found in most APS-1 patients (7 of 8) with respiratory involvement of varying severity - with fatal outcomes in some cases. We demonstrate that KCNRG expression is mainly restricted to the epithelial cells of terminal bronchioles. These findings have significance for clinicians who care for patients with APS-1 and provide a tool to define and investigate the possible pulmonary autoimmunity in APS-1 and thereby distinguish it from concurrent obstructive lung disease or lower respiratory tract infections. The terminal bronchiole is a previously unrecognized autoimmune target in APS-1.

Symptoms displayed were quite variable (**Table 1**). Patients 6-8 had relatively mild symptoms, well controlled by inhaled beta₂-agonists, inhaled glucocorticoid and mucolytic drugs, and may go unnoticed in the context of APS-1 where several disabling disease components can mask less obvious symptoms. In contrast, four patients (patients 1-4, **Table 1**) had severe respiratory symptoms, initially asthma-like but evolving into severe obstructive lung disease with radiological signs of bronchiectasis. Two died from pulmonary disease at the age of 18 and 37 years, another patient is oxygen-dependent at the age of 18, but patient 1 displayed dramatic improvement when treated with mycophenolate mofetil immunosuppression. In patients 1, 2 and 4, the relationship between the respiratory symptoms and APS-1 was not considered initially even when cystic fibrosis had been ruled out. All of these four patients had high titer autoantibodies to KCNRG and early knowledge of this may have influenced their management. KCNRG autoantibodies are however, discordant with pulmonary manifestations in two APS-1 patients (Patients 5 and 9). Patient 5 was negative for KCNRG autoantibodies when her initial respiratory symptoms appeared. A presumed lymphocytic interstitial pneumonia (LIP) had remitted after hydroxychloroquine treatment. However, in addition to APS-1, this patient had recurrent pulmonary bacterial infections due to antibody deficiency. She responded well to intravenous immunoglobulin replacement therapy (IVIG), but has had recurrent unexplained respiratory symptoms thought to be due to LIP. This treatment may have altered this patient's pulmonary presentation and/or masked the detection of autoantibodies. To our knowledge, this patient is the only case of APS-1 on IVIG therapy. Patient 9 was positive for KCNRG autoantibodies but has no respiratory symptoms or pathological signs on chest X-ray, spirometry, or plethysmography. In addition, it should be taken into consideration that respiratory disease in this report was defined from patients' self-reported symptoms and not by prospective lung function tests. It may therefore be possible that some additional cases of patients with undetected respiratory disease may exist in our cohort. These possible cases apparently do not show autoantibody response to KCNRG.

The absence of a perfect correlation between presence of KCNRG autoantibodies and respiratory manifestation is not surprising. It parallels other autoantibody responses in APS-1 and other autoimmune conditions where only a fraction of autoantibody-positive individuals manifest the clinical disease component at any time point^{19,20}. There may also be heterogeneity of the immune response in APS-1 and other pulmonary autoantigens could be targeted. Since we could only test a single serum from each patient, it is impossible to rule out fluctuation of KCNRG autoantibodies over time. Alternatively, APS-1 patients may present with confounding respiratory symptoms due to common intercurrent diseases (asthma and infections) or candida infections.

Although pulmonary autoimmunity hitherto has not been considered as a component of APS-1 in humans^{11,21}, the animal model for APS-1, *Aire* deficient mice display pulmonary pathology of variable severity depending on the background strain. *Aire* deficient mice on C57BL6 and Balb/c background display modest pulmonary disease while *Aire* deficient mice on NOD and SJL background strain have severe and fatal lung pathology comparable to the histological appearances in our APS-1 patients⁹. Phenotypic variability may therefore depend on genetic background in humans.

Is KCNRG is a valid candidate bronchial autoantigen? Northern blotting and quantitative real-time PCR analysis demonstrate predominant expression in lungs and immunohistochemistry localize the KCNRG protein to epithelial cells of small bronchioles. Two human splice variants of KCNRG encoding 31 and 26 kDa isoforms have been characterized by ourselves and others²². KCNRG has a homology to the cytoplasmic tetramerization domain of voltage gated potassium channels and KCNRG inhibits potassium fluxes *in vitro* suggesting that KCNRG may function as a potassium channel regulating protein²². We have experimentally confirmed the tendency of KCNRG to form tetramers *in vitro* (Supplementary Figure 2). Although the exact role of KCNRG in the lung remains to be determined, a role of potassium channels in histamine-induced bronchoconstriction and plasma exudation has been postulated and drugs interfering with potassium channels have been proposed to treat bronchoconstriction^{23,24}. It is also well recognized that autoantibodies to calcium and potassium channels can cause autoimmune disease such as the Lambert-Eaton Myasthenic Syndrome²⁵.

We have identified KCNRG, a putative potassium channel regulating protein expressed in bronchial epithelial cells, as an autoantigen in APS-1 associated with pulmonary manifestations. We report pulmonary autoimmunity as a disease component in APS-1 with a potentially fatal outcome. Early recognition of pulmonary autoimmunity and its distinction from asthma and recurrent bacterial infections is important, since the autoimmune bronchiolitis in APS-1 may respond well to immunosuppression. Our findings also highlight APS-1 as a condition, which provides an important opportunity to study autoimmunity in the lungs.

Materials and Methods

Ethics Approval

Informed written consent was obtained for all participants. Ethics committee approval was obtained from the Uppsala University (Permit UPS-02-415).

Patients and Sera

Serum samples were analyzed from 110 APS-1 patients (10 Swedish, 19 Norwegian, 59 Finnish, 18 Italian, 4 French and 1 from UK) with at least two of the major clinical components of APS-1 (Addison's disease, hypoparathyroidism, and chronic mucocutaneous candidiasis). The following diagnostic criteria were used: mucocutaneous candidiasis: candidal infections in the oral mucosa, skin or nails for more than 3 months. Hypoparathyroidism: Subnormal plasma calcium concentration (<2.15 mmol/L) and supranormal plasma phosphate concentration together with normal or low PTH concentrations, and normal renal function. Addison's disease: subnormal serum cortisol together with elevated plasma ACTH concentrations, or failure to reach s-cortisol of 550 nmol/L at 30 or 60 min of an ACTH stimulation test. The majority of the patients diagnosed with Addison's disease also displayed specific 21-hydroxylase autoantibodies. The majority of the patients were also demonstrated to have typical mutations in the *Aire* gene (102 of the 110 patients). All of the 9 patients with KCNRG autoantibodies had typical mutations in the *Aire* gene. Detection of respiratory symptoms: Due to the high number of included patients from several centers in six different countries, we could not systematically perform lung function examination on the entire cohort. Hence, respiratory symptoms described here were defined from patient self-report

of dyspnoea or cough, leading to relevant pulmonary work-up to exclude other causes of respiratory symptoms. Detailed information on each of the patient's respiratory symptoms is included in the Results section of the manuscript. Control sera were obtained from patients with: allergic asthma (n=24), non-allergic asthma (n=24), chronic obstructive pulmonary disease (COPD) (n=45), Sjögren's syndrome with respiratory symptoms (n=8), Addison's disease (n=30), type 1 diabetics (n=30), and healthy blood donors (n=91).

Construction and Screening of cDNA Expression Library

Messenger RNA was isolated from bovine tissue, obtained at a local abattoir. A cDNA expression library was constructed in the λ -ZAP Express vector (Stratagene, La Jolla, CA). The library was immunoscreened with serum from an APS-1 patient (Patient 6, **Table 1**) as previously described¹². Isolated clones were sequenced and their DNA and deduced amino acid sequences were analyzed using the Basic Local Alignment Sequence Tool (BLAST)¹³.

Generation of ³⁵S Labeled Human KCNRG and Immunoprecipitation/ Radioimmunoassay for KCNRG Autoantibodies

The KCNRG-encoding clone, isolated by immunoscreening of the cDNA library, was used as template for coupled *in vitro* transcription, translation and labeling with ³⁵S-Methionine using the TnT system (Promega, Madison, WI)¹⁴. Autoantibody reactivity against the clones was determined by immunoprecipitation followed by analysis of the immunoprecipitates on SDS-PAGE, and/or evaluation of the precipitated radioactivity on an automated beta counter as previously described^{15,16}.

Expression Analysis by Quantitative PCR and Northern Blot

Complementary DNA from normal human tissues obtained from BD Biosciences (Palo Alto, CA, USA) were normalized and used as templates for quantitative PCR analysis on an iCycler MyiQ (Bio-Rad, Hercules, CA). Primer sequences, PCR conditions and Conditions for the Northern blotting is provided in the Supplementary appendix.

KCNRG Antiserum Generation and Immunoblotting

An antiserum against KCNRG was raised by immunization of rabbits with the peptide LPPQRPSYHDLVFQC, present in both human and bovine KCNRG and affinity-purified on a peptide column. Specificity was confirmed by immunoblotting with bovine lung total protein extract and by absorption studies in which the reactivity was blocked by preincubation with the peptide used for immunization.

Immunohistochemistry

Samples of bovine lung were fixed and paraffin embedded. Sections of 4 μ m thickness were deparaffinized, microwave treated, blocked and incubated overnight at 4°C with the KCNRG antiserum (dilution 1:1000). The slides were then washed, exposed for 30 minutes to a biotinylated secondary antibody and developed using the VECTASTAIN ABC system (Vector Laboratories, Burlingame, CA) and ChemMate DAKO Envision Detection kit (DAKO, Glostrup, Denmark). Negative control slides were used for comparison.

Immunofluorescence and Laser-Scanning Confocal Microscopic Analysis

Cryosections (6 μ m) of bovine lung tissue were air dried, blocked and incubated with APS-1 patient's sera with KCNRG reactivity (dilution 1:400). The slides were incubated with FITC conjugated secondary antibodies (dilution 1:200) for 30 min. Slides were analysed on a Zeiss LSM 510 confocal microscope. Sera from healthy blood donors and from APS-1 patients without KCNRG-specific autoantibodies were used as negative controls.

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Table 1. Description of the APS-1 patients with respiratory symptoms or KCNRG antibodies								
Patient # (country of origin)	Age of onset of pulmonary manifestations	Initial pulmonary symptoms	Pulmonary function tests/ X-rays/ pulmonary histology	Management	Outcome	Antibodies to KCNRG	APS-1 manifestations	Aire gene mutation
1 (France)	Childhood (≈5 years of age)	Obstructive symptoms with frequent exacerbations	Obstructive pulmonary disease/ ground glass opacities, bronchiectasis/ 12 yr: bronchiolopathy and peribronchiolar inflammatory infiltrate	Steroids, azathioprine, mycophenolate mofetil	Steroid dependence: marked improvement on mycophenolate mofetil	+	Candidiasis, hypoparathyroidism, Addison's disease, alopecia, pernicious anemia, exocrine pancreas insufficiency	62C>T/ 1096-1G>A
2 (Italy)	Childhood	Several lower respiratory tract infections from 5 years of age	Obstructive pulmonary disease/ 10 yr: bilateral bronchiectasis	Antibiotics, supportive care	Death at 18 yr due to cor pulmonare and terminal respiratory failure.	+	Candidiasis, hypoparathyroidism, Addison's disease, pernicious anemia, intestinal dysfunction/malabsorbtion	607C>T/ 769C>T
3 (Finland)	Childhood	Asthma-like symptoms	Obstructive and restrictive pulmonary disease/ bronchiectasis/ 10 yr: bronchiolopathy, bronchiolitis obliterans	Steroids	Severe respiratory failure	+	Candidiasis, hepatitis, intestinal dysfunction/ malabsorption, Addison's disease	769C>T/ 769C>T
4 (France)	Childhood	Chronic cough	Obstructive then restrictive pulmonary disease/ 20 yr: bronchiectasis/ 34 yr: peribronchiolar inflammatory infiltrate	Antibiotics, supportive care	Exacerbations with recurrent pulmonary infections; death at 37 yr of chronic respiratory failure	+	Candidiasis, hypoparathyroidism, Addison's disease, hepatitis	964del13/ 964del13
5 (United Kingdom)	Childhood (11 yr)	Cough, dyspnea, thoracic pain	Restrictive pulmonary disease with decreased DL,CO/ bilateral interstitial infiltrate and peribronchial ground-glass opacity / lymphocytic bronchiolitis	Hydroxychloroquine from 11 to 18 yr	Improvement at the age of 20 yr	-	Candidiasis, hypoparathyroidism, Addison's disease, hepatitis, ectodermal dysplasia, specific antibody deficiency (failure to respond to polysaccharide vaccine and recurrent bacterial infections), on intra-venous immunoglobulin	964del13/ 964del13
6 (Sweden)	Young adulthood	Airway hyperresponsiveness and obstructive symptoms	N.D.	Inhaled terbutaline and budesonide, acetylcysteine	Mild to moderate symptoms of bronchial hyperresponsiveness.	+	Candidiasis, hypoparathyroidism, Addison's disease, hypogonadism, alopecia, vitiligo	769C>T/ 769C>T
7 (Sweden)	Young adulthood	Airway hyperresponsiveness.	N.D.	Inhaled terbutaline, acetylcysteine	Improvement	+	Candidiasis, hypoparathyroidism, Addison's disease, hypogonadism, hepatitis, intestinal dysfunction/malabsorbtion	769C>T/ 769C>T
8 (Sweden)	Young adulthood	Airway hyperresponsiveness. Respiratory symptoms. Infection induced exacerbations.	N.D.	Inhaled terbutaline, acetylcysteine	Improvement; occasionally, mild respiratory symptoms	+	Candidiasis, hypoparathyroidism, Addison's disease, hypogonadism, intestinal dysfunction/malabsorbtion, hepatitis, gastritis	M388fsX36 / C62>T
9 (Norway)	N.A.	No respiratory symptoms	N.D.	N.A.	No respiratory symptoms	+	Candidiasis, hypoparathyroidism, Addison's disease, hypogonadism, intestinal dysfunction/malabsorbtion	967-979del13/ 967-979del13

N.D. Denominates not done. N.A. denominates not applicable. Seven of eight patients with respiratory symptoms had antibodies to KCNRG, while KCNRG antibodies were positive in only one APS-1 patient without respiratory symptoms.

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Table 1

Please find the enclosed PDF-file for the Table.

Figure legends

Figure 1: Radiological and Histological Aspects of Pulmonary Involvement in APS-1 Patients **A)** Pulmonary CT scan in patient 1 at the age of 11 years: peribronchial ground glass opacities **B)** Pulmonary CT scan in patient 1 at the age of 16 years, on immunosuppressive treatment with mycophenolate mofetil; the peribronchiolar abnormalities are improved. **C and D)** Histological appearance of lung biopsy in patient 1 at the age of 11 years (**C**, 10X magnification, **D**, 40X magnification): peribronchiolar lymphoid infiltrate. **E)** Pulmonary CT scan in patient 3 showing established bronchiectasis. **F)** Lung biopsy on patient 3 showing bronchiolitis obliterans organizing pneumonia. **G and H)** Histological appearance of lung biopsy in patient 4, at the age of 35 years, two years before his death from chronic respiratory failure (**G**, 10X magnification, **H**, 40X magnification); showing severe peribronchiolar infiltrate.

Figure 2: Autoantibody Reactivity to KCNRG and Tissue Expression of KCNRG messenger RNA. **A)** Comparison of KCNRG autoantibody titers in sera from APS-1 patients, patients with different pulmonary disorders or other autoimmune disorders, and in sera of healthy blood donors. The assay for detection of autoantibodies is described in Methods. The dashed line indicates a cut of value of 0.41, which is the upper level of normal, defined as the mean results obtained for the healthy blood donors +3 SD.

B) Expression of KCNRG mRNA in adult human tissues as measured by quantitative PCR, demonstrating that the expression of KCNRG is mainly restricted to pulmonary tissue. Please note the non-continuous y-axis.

Figure 3. Distribution of KCNRG Protein in Bovine Lung. A-C). Immunohistochemistry on 4 μ m paraffin embedded sections, using affinity-purified anti-KCNRG rabbit antiserum. **A)** Background staining without the primary antibody. **B)** Antiserum used in dilution 1:1000. **C)** Antiserum in dilution 1:1000 pre-absorbed with 20 nmol of the peptide used for the immunization. **D-K)** Immunofluorescence on 6 μ m cryosections of lung, using patient and control serum samples. The blue background is from DAPI that stains nucleoli. FITC conjugated goat-anti-human IgG secondary antibody (green) was used. **D)** Background staining when no primary serum is used. **E-G)** Staining with three APS-1 patients' sera with KCNRG reactivity. **H-I)** Staining with two APS-1 sera without KCNRG reactivity. **J-K)** Staining with sera from two healthy blood donors.

Figure 1

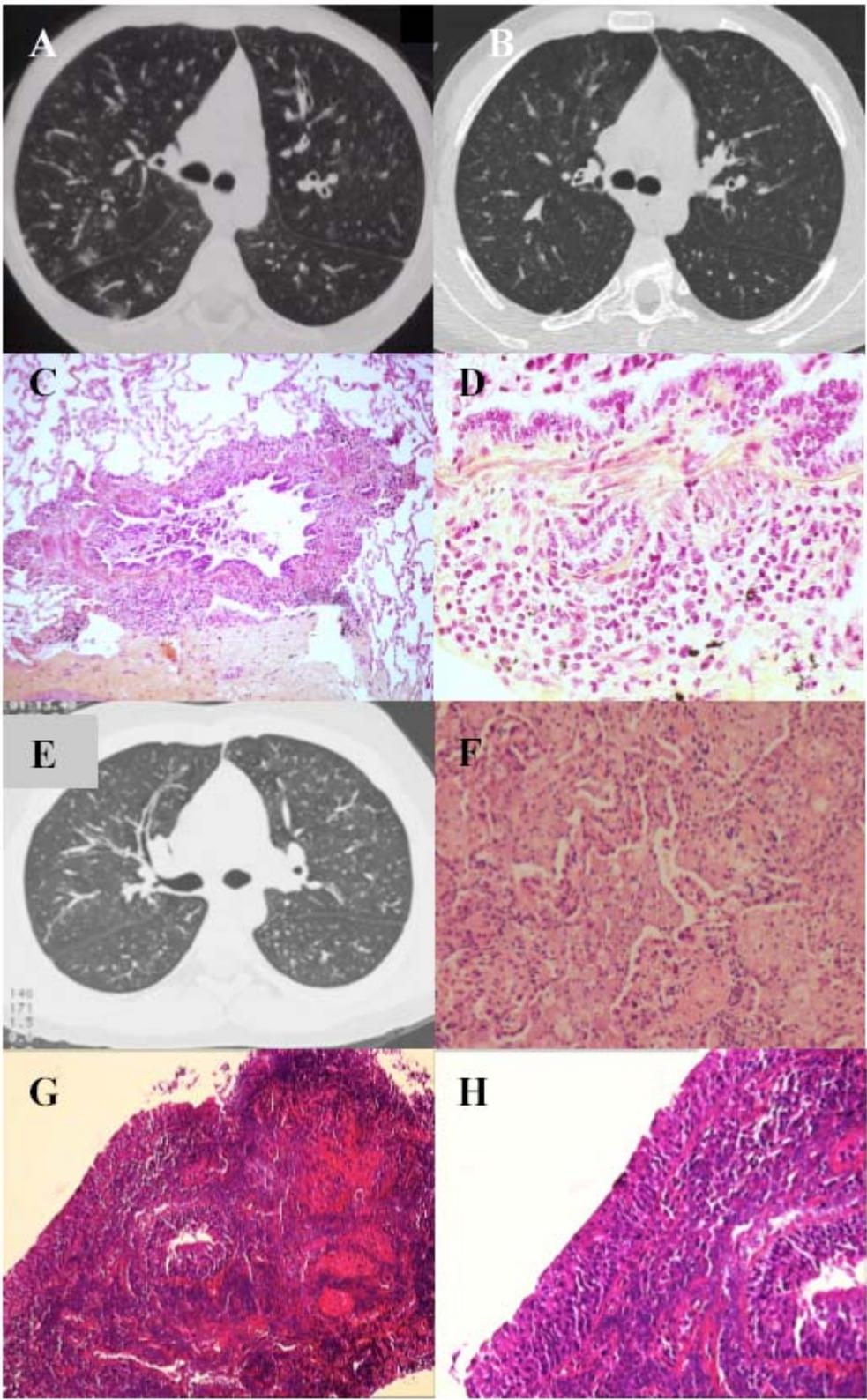


Figure 2A

KCNRG-specific autoantibodies in different pulmonary- or autoimmune disorders

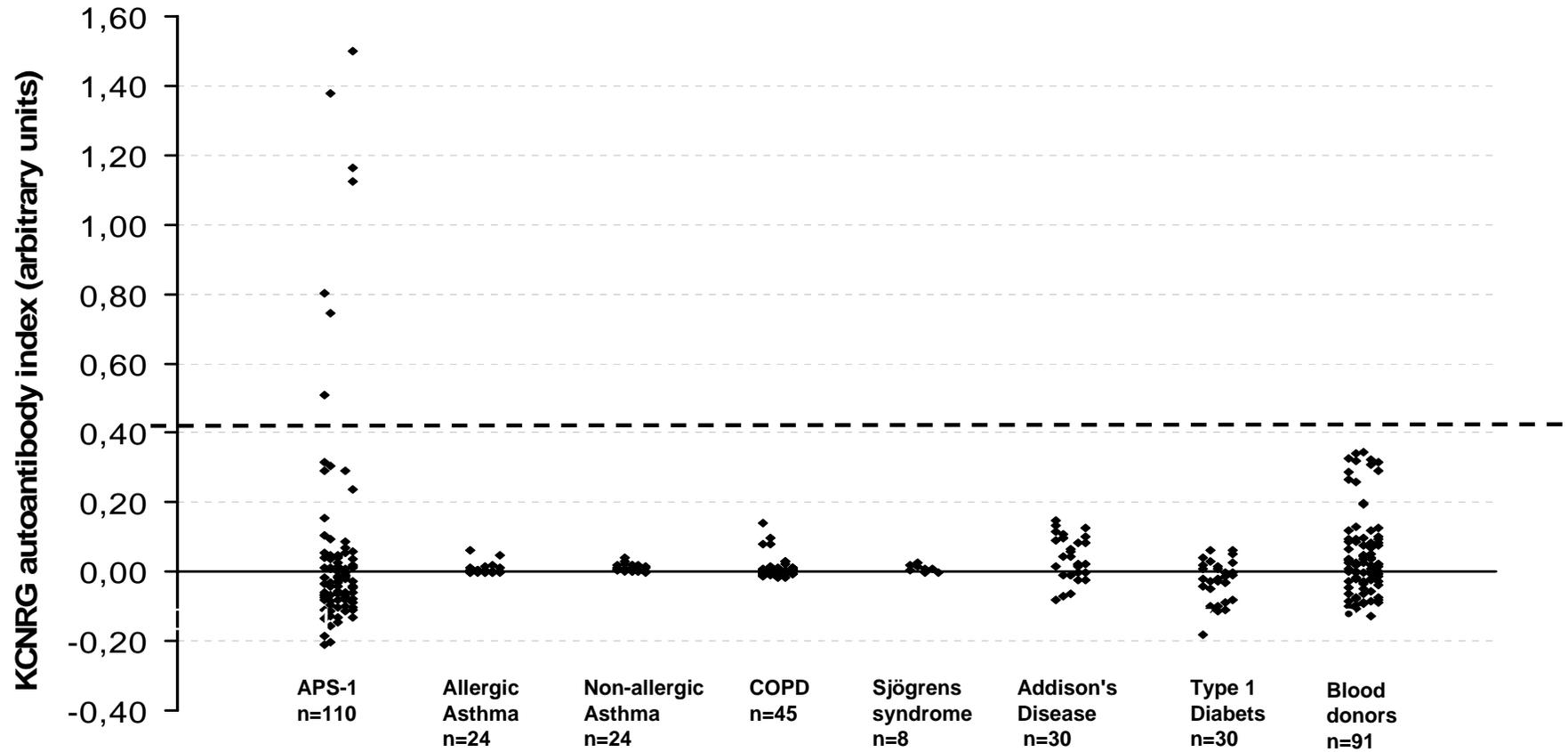


Figure 2B

Distribution of KCNRG mRNA in Human Tissues

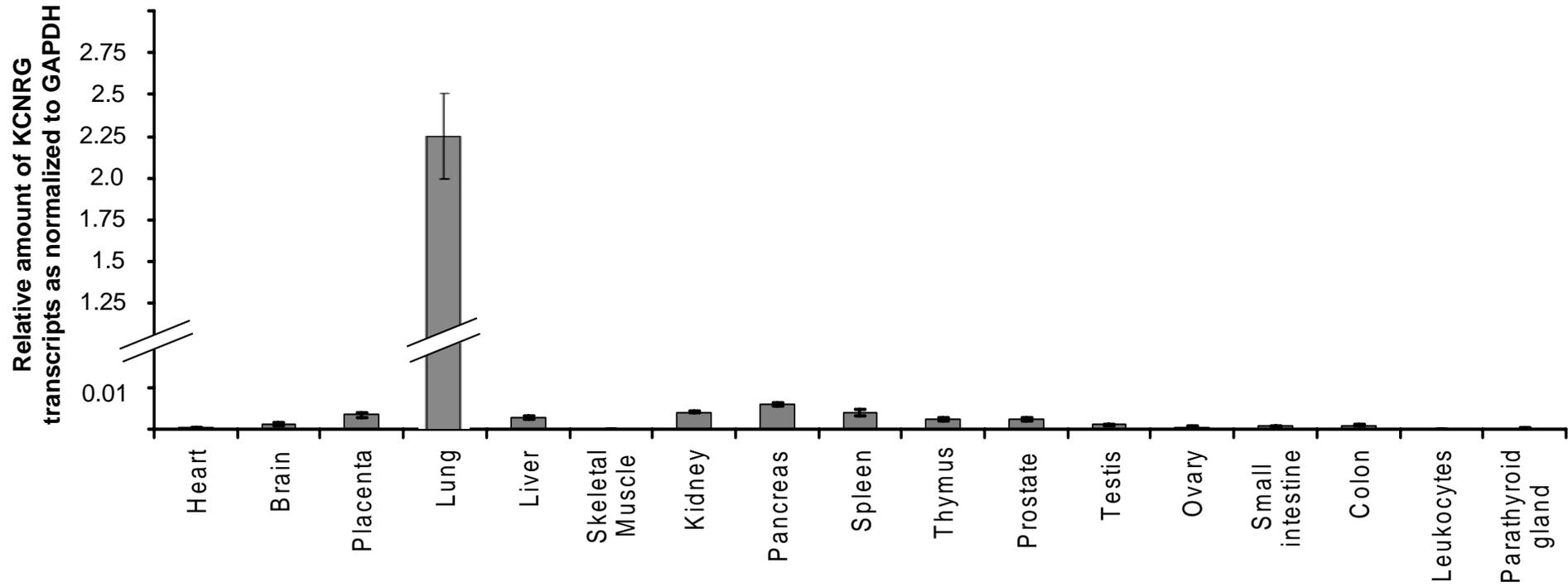
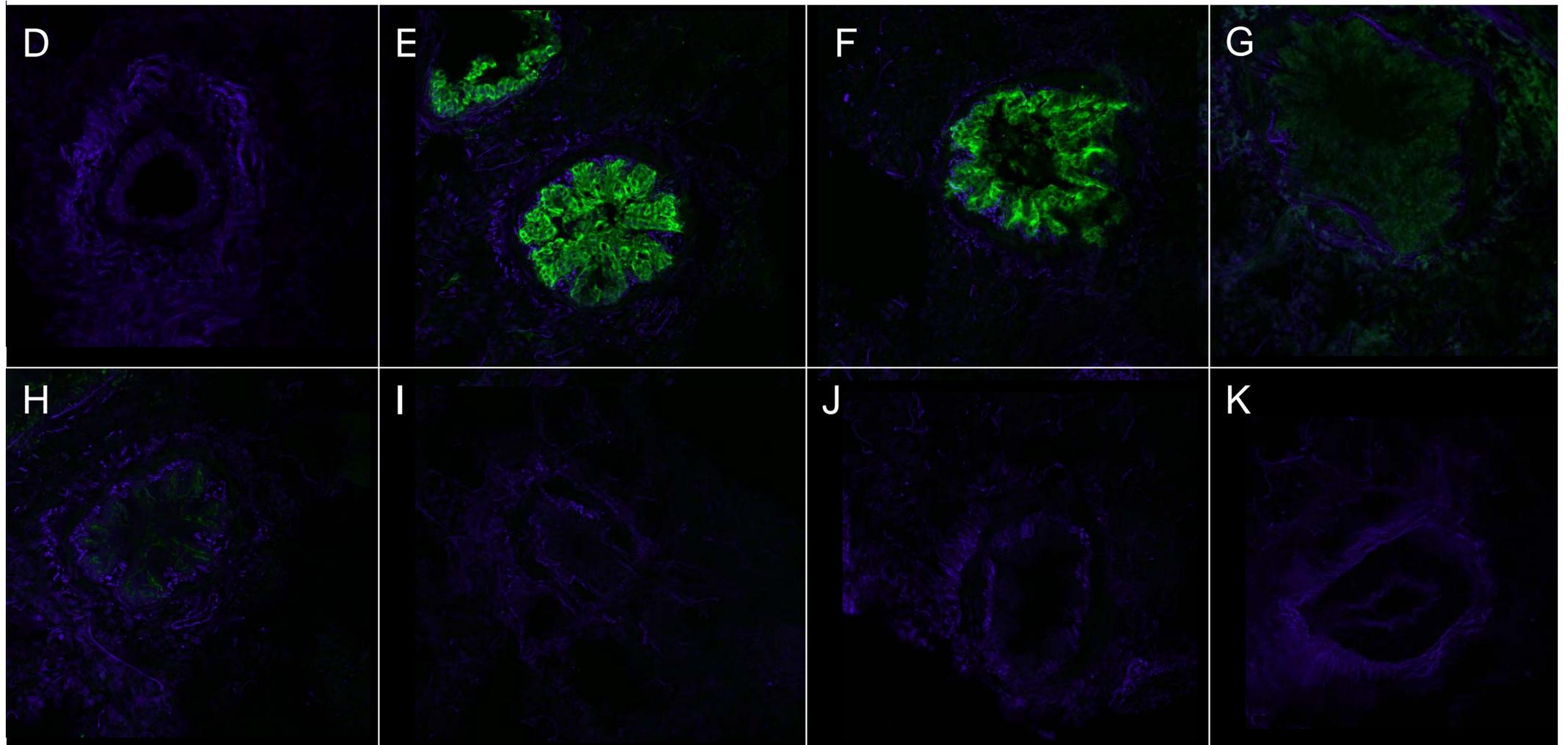
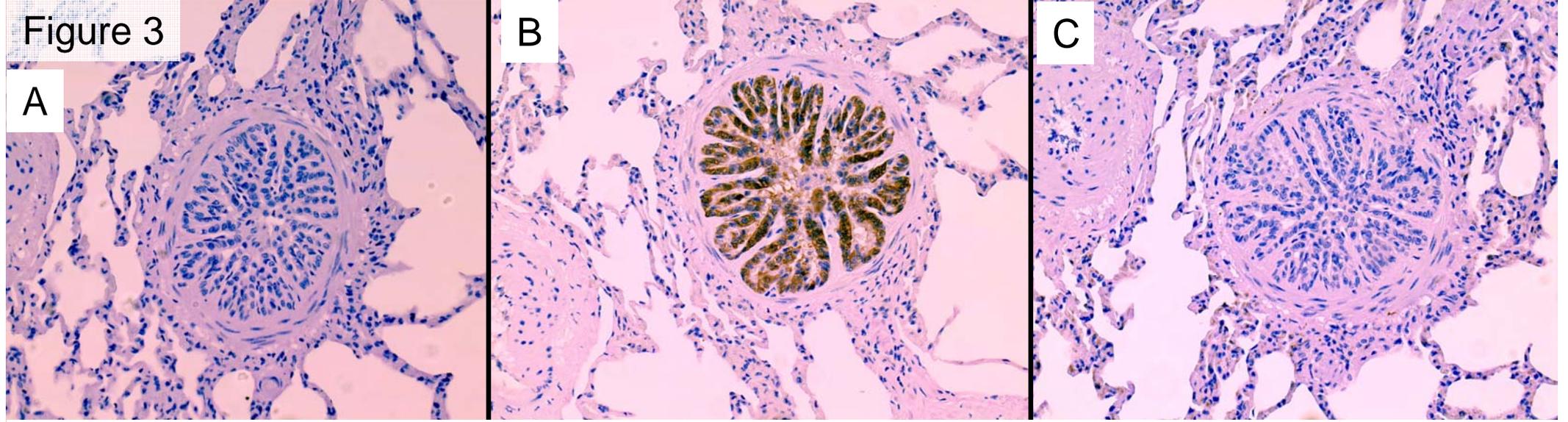


Figure 3



Supplementary Appendix

Methods

Statistical Analysis

Fisher's exact test was used to determine the association of the KCNRG autoantibodies to different clinical manifestations of APS-1 in patients with the syndrome.

DNA Sequencing and Analysis

Isolated clones from immunoscreening of the cDNA library were subjected to *in vivo* excision of the pBK-CMV phagemid and automated BigDye Terminator cycle sequencing on an ABI377 sequencing machine (Applied Biosystems, Foster City, CA, USA). The human full length clones for 26kDa and 31kDa isoform of KCNRG IMAGE clones 4690662 and 5207702 respectively were obtained from the UK HGMP Resource Centre (Cambridge, UK). DNA and deduced amino acid sequences were analyzed using the Basic Local Alignment Sequence Tool (BLAST) and Conserved Domain Database searches at www.ncbi.nih.gov. Amino acid sequences were also analyzed by use of the PSORT II program at psort.nibb.ac.jp. ClustalW alignments were performed at www.ebi.ac.uk. Computations of the theoretical molecular weights were performed by use of the Compute pI/Mw tool at us.expasy.org.

Radioimmunoassay for KCNRG Autoantibodies and Immunoprecipitation Experiments

The bovine KCNRG cDNA clone and the two human KCNRG full length splice variants were used as templates for coupled *in vitro* transcription/translation using the TnT system (Promega, Madison, WI, USA). For analysis of antibody reactivity against *in vitro*-translated KCNRG, a 96-well immunoprecipitation assay was used, essentially as described (References 12-13 in the article). All sera were analyzed in duplicates. The immunoprecipitated radioactivity was evaluated in a liquid scintillation counter (Wallac 1450 MicroBeta). The screening serum (patient 6) and a healthy blood donor serum served as positive and negative controls, respectively. For each reaction, 20000 cpm of radiolabeled KCNRG and 2.5 μ l of serum were used in a final volume of 50 μ l. An anti-KCNRG reactivity index was calculated for each serum as follows: $(\text{cpm of unknown sample} - \text{cpm of negative control}) / (\text{cpm of positive control} - \text{cpm of negative control}) * 100$. Upper level of normal was defined as the mean results obtained for the healthy blood donors included +3 SD and is indicated as a dashed line in the scattergram. The intra-assay and inter-assay coefficient of variations were 10 % and 14 %, respectively.

For the immunoprecipitation experiments in **Supplementary Figure 3**, immunoprecipitation was performed in microcentrifuge tubes. Typically, 20 000 cpm of the *in vitro* translation product was incubated with 2.5 μ l serum in a final volume of 100 μ l in 20 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% sodium azide and 1% Tween-20. After binding to Fast Flow Protein A Sepharose (GE-healthcare, Uppsala, Sweden) immune complexes were washed six times and bound proteins analyzed by SDS-PAGE followed by autoradiography using Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA).

Preabsorption Experiments

To confirm the specificity of APS-1 patient sera for KCNRG, absorption studies were performed using serum samples from two patients (patients 6 and 7 described in Table 1) with KCNRG autoantibodies as detected in the autoantibody assay. Serum from these patients were diluted (1:800) in

20 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% sodium azide to a final volume of 800 μ l and incubated over night with either 20 μ l PBS (as a negative control), or 20 μ l *in vitro* transcribed and translated and 35 S-radiolabeled KCNRG (\approx 200 000 cpm), or 20 μ l *in vitro* transcribed and translated and 35 S-radiolabeled Luciferase (\approx 1 000 000 cpm). After over night absorption, the preabsorbed serum samples were tested using immunofluorescence on bovine lung sections as described under methods in the manuscript.

Immunohistochemistry on Tissue-Multi-Array (TMA) from the Swedish Human Protein Atlas Project

A TMA slide as described on www.proteinatlas.org was stained with the KCNRG antiserum in dilution (1:2000) as described under Methods/Immunohistochemistry in the article. After the staining, counterstaining with hematoxylin and eosin was performed.

Northern Blotting

A 701 bp EcoRI fragment was excised from the KCNRG 31 kDa myc/his expression construct (see below) and used as template for synthesis of a 32 P-labeled DNA probe by random priming with the Strip-EZ DNA kit (Ambion, Austin, TX, USA). The probe was purified by size exclusion chromatography on a NICK column (Amersham Biosciences, Uppsala, Sweden). A human 12-lane multiple tissue northern blot (BD Biosciences) was hybridized with the probe (\sim 10⁶ cpm/ml) in ULTRAhyb solution (Ambion). After washing, blots were analyzed by phosphorimaging.

Quantitative Real-time PCR

Real-time PCR was carried out on an iCycler MyiQ (Bio-Rad, Hercules, CA, USA) and analyzed using the software of the manufacturer. In a total volume of 25 μ l, each reaction contained 2 μ l of the cDNA preparation provided in normalized multiple-tissue cDNA panels or normal human parathyroid cDNA, 300 nM of each primer pairs (KCNRG qPCRfw + KCNRG qPCRrev or GAPDHf + GAPDHR) and 12.5 μ l iQTM SYBR[®]-Green supermix (Bio-Rad). Cycling conditions were as follows: 95°C for three minutes, followed by 50 cycles of 55°C for 15 seconds and 60°C for 30 seconds. Melt curve analysis and direct sequencing of the PCR product verified the specificity of the amplification product. The PCR analysis included a standard curve and non template negative controls. The relative amounts of the endogenous control glyseraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and the gene of interest mRNA in each sample could be deduced from the standard curves.

Primer Sequences

Primers were designed using the Beacon designer software (Premier Biosoft International, Paolo Alto) on the human genomic and cDNA sequence for 31kDa KCNRG. The following oligonucleotide primers (CyberGene AB, Huddinge, Sweden) were used for quantitative real time PCR: KCNRG qPCRfw, CAGGGAGGATTACAGTGTTTACAG; KCNRG qPCRrev, TTTATAGAAACATACCTGA CTCC ; GAPDHfw, AGGGCTGCTTTTAACTCTGGTAA; GAPDHrev, CATATTGGAAC ATGTAAACCATGTAGTT

Hydrodynamic Parameters and Molecular Weight of KCNRG

The sedimentation coefficient of *in vitro*-translated 35 S-methionine-labeled bovine KCNRG cDNA was estimated by sucrose gradient sedimentation. Ten μ l translation product was loaded on a gradient of 10-40% sucrose in PBS together with 100 μ g bovine cytochrome c (sedimentation coefficient 1.86 S) and 0.5 μ g rabbit IgG (6.7 S), which were used as internal

standards along with the hemoglobin (4.4 S) present in the translation reaction. The tubes were centrifuged in a Beckman SW50.1 rotor at 50,000 rpm, at +4°C for 18 hours. Fractions were collected from the bottom of the tube and analyzed by scintillation counting, SDS-PAGE followed by phosphorimaging, spectrophotometry at 414 nm, to detect hemoglobin and cytochrome c, and direct ELISA to detect rabbit IgG. Radioactive bands in SDS-polyacrylamide gels were quantified using ImageQuaNT software (Molecular Dynamics).

The Stokes radius (R_s) was estimated by size exclusion chromatography on a Superdex 200 HR 30/10 column, connected to a BioLogic HR chromatography system with UV absorbance detection at 280 nm and a flow rate of 0.3 ml/min. The column was equilibrated with 20 mM Na-Hepes, containing 200 mM NaCl, pH 7.0 and 15 μ l of the *in vitro* translation reaction was injected. The column was calibrated and the chromatograms analyzed as previously described (15), using the following standard proteins in two separate calibration runs applying four standards for each run: catalase (R_s 5.2 nm), transferrin (3.5 nm), ovalbumin (3.05 nm), myoglobin (2.11 nm), thyroglobulin (8.5 nm), apoferritin (6.1 nm), BSA (3.55 nm) and cytochrome C (1.7 nm). Blue dextran and acetone were used to determine the void volume (V_0) and the total liquid volume inside the column (V_t), respectively. To detect the KCNRG translation product, fractions were analyzed by scintillation counting.

Standard curves for sedimentation coefficients and R_s values were generated by linear regression analysis using Microsoft Excel 2002 software. The molecular weight of *in vitro*-translated bovine KCNRG was estimated from combined gel filtration and gradient sedimentation using the Svedberg equation:

$$M_r = SN_0(6\pi\eta R_s)/(1-v_2\rho) = 4,204S'R_s'$$

where M_r is molecular weight, S is the sedimentation coefficient, N_0 is Avogadro's number, η is the viscosity of the solvent (0.01 poise), v_2 is the partial specific volume of the protein (assumed to be 0.73 cm³/g) and ρ is the density of the solvent (1.0). For the numerical formulation on the right, S' is in Svedberg units and R_s' is in nm.

Immunoprecipitation and Immunoblotting

Bovine lung tissue was homogenized in 20 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% sodium azide and 1% Triton X-100. After centrifugation, the supernatant was used for immunoblotting with the specific rabbit antiserum. As a control, non-radioactive *in vitro*-translated bovine KCNRG was used. 500 μ l of the lysate was diluted 1:1 with Laemmli Sample Buffer (cat# 161-0737, Bio-Rad laboratories Hercules, CA) supplemented with 50 mM dithiothreitol and denatured at 95°C for 2 minutes and separated on a 12% Criterion SDS-PAGE Tris-HCl gel (cat#345-0019, Bio-Rad laboratories Hercules, CA) and transferred onto Nitrocellulose membrane (cat#162-0167 Bio-Rad laboratories Hercules, CA). Running and transfer conditions according to the manufacturer's instructions (Bio-Rad). The blot was blocked with 5% dry milk for 30 minutes and incubated with the primary antibody (1:400-1:2000) at 4°C overnight. After washing, bound primary antibodies were detected by a HRP conjugated secondary antibody using an ECL kit (Cat#RPN2132 GE Healthcare) according to the manufacturer's instruction

Results

In Silico Analysis of KCNRG and Identification of KCNRG Orthologs

BLAST searches of the nr database at NCBI revealed that the ortholog of this transcript is encoded on chromosome 13q14 in human and on chromosome 14 C3 in mouse. Unspliced transcripts of the 5' end have been reported by Bullrich *et al.* who named the gene CLLD4 (Bullrich, F. *et al.* (2001) cancer Research **61**, 6640-6648). By PCR amplification using specific primers, we were able to isolate two KCNRG splice variants from lung cDNA derived from both human and mouse (GenBank accession numbers AY190921-2 and AY491736-7). In human, these variants correspond to the human IMAGE clones 4690662 and 5207702, of which the full sequences have been published by Ivanov *et al.* who used the gene name KCNRG (reference 18 in the article). In human, these splice variants are generated by alternative usage of a 98 bp middle exon that introduces a frame shift resulting in a smaller translation product. This exon is part of an Alu sequence of the Sp superfamily (GenBank accession number U14572, 90% nucleotide identity over 285 bp by BLASTN alignment). The two human splice variants encode proteins with predicted masses of 31 and 26 kDa, respectively. In mouse, two alternative splice sites exist at the 3' end of exon 1, resulting in transcripts differing 34 bp in length, of which the longer variant contains a premature stop codon. By searching the EST database we also identified an ortholog transcript from *Silurana tropicalis* (IMAGE clone 5336560), of which we sequenced the entire open reading frame (GenBank accession number AY190924).

The N-terminal region of KCNRG of all species contains a K₄ tetra or BTB/POZ domain motif (pfam02214 and smart00225, respectively), as identified by searches in the Conserved Domain Database. There is also a conserved leucine zipper motif in the 31 kDa isoform, as identified by the PSORT II program, located more to the C terminus. The frog sequence also contains two overlapping nuclear localization signal (NLS) motifs at positions 246-249 and 247-250, respectively.

Analysis of Autoreactivity Against Different Splice Variants of KCNRG

To determine whether the autoantibodies against KCNRG distinguished between the 31 kDa and 26 kDa isoforms of the human protein, KCNRG positive APS-1 sera, KCNRG negative APS-1 sera and healthy blood donor sera were used in immunoprecipitation of ³⁵S-labeled *in vitro*-translated bovine KCNRG, human 31 kDa isoform of KCNRG and human 26 kDa isoform of KCNRG proteins followed by analysis on SDS-PAGE and autoradiography. As shown in **Supplementary Figure 3**, sera from APS-1 patients with autoantibodies directed against KCNRG immunoprecipitated both the 26kDa and 31kDa human splice variants of KCNRG as well as bovine KCNRG, identically. This demonstrates that all these three isoforms of KCNRG contain the epitope recognized by patients' autoantibodies.

Expression Analysis of KCNRG mRNA by Northern Blot.

Tissue distribution of KCNRG was investigated by northern blotting. A multiple tissue northern blot was hybridized with a cDNA probe corresponding to amino acids 42-273 of the 31 kDa isoforms, thus lacking part of the K₄ tetra/BTB/POZ domain sequence, to reduce possible cross-hybridization (**Supplementary Figure 1**). In the lung lane, a specific band at ~1.35 kb is seen. In most of the lanes, there is a band at ~7.5 kb, presumably due to cross-hybridization with transcripts of the overlapping or existence of additional splice variants.

Illustration of KCNRG's Appearance as Monomers and Tetramers *in vitro*

As the N terminus of KCNRG contains a K_{tetra}/BTB/POZ domain motif, which is known to mediate oligomerization in other proteins such as ion channels, we investigated the hydrodynamic characteristics of *in vitro*-translated KCNRG. Bovine KCNRG (which gave the best yields of translation product) was subjected to ultracentrifugation on a sucrose gradient and size exclusion chromatography, and fractions were analyzed. As shown in **Supplementary Figure 2A**, the *in vitro*-translated protein was found to give rise to two distinct sedimentation peaks with apparent sedimentation coefficients of approximately 2.7 S and 6.4 S, respectively. A similar profile was obtained in the absence of cytochrome c and IgG, indicating that these proteins do not interfere (not shown). Size exclusion chromatography also resulted in two separate peaks corresponding to estimated R_s values of 3.1 and 5.4 nm, respectively (**Supplementary Figure 2B**). From these sedimentation coefficients and R_s values, the molecular weight of the two forms of KCNRG could be estimated to 36 and 146 kDa, respectively. Given the predicted molecular weight of the polypeptide monomer (30 kDa), these values indicate that KCNRG exist as tetramers and monomers *in vitro*, although complex formation with other proteins in the translation reaction cannot be excluded by these experiments.

APS-1 Disease Component	Number of Patients with Disease component/total (Percent)	Number with antibodies to KCNRG/total		P value
		With disorder	Without disorder	
Self-reported pulmonary symptoms	8/110 (7%)	7/8 (88 %)	1/102 (1%)	<0.0001
Candidiasis	105/110 (95%)	8/105 (8%)	0/5 (0%)	0.45
Hypoparathyroidism	93/110 (85%)	7/93 (8%)	1/17 (6%)	0.54
Addison's disease	87/110 (79%)	7/87 (8%)	1/23 (4%)	0.31
Hypogonadism	32/110 (29%)	3/32 (9%)	5/78 (6%)	0.78
Alopecia	38/110 (35%)	3/38 (8%)	5/72 (7%)	0.51
Vitiligo	22/110 (20%)	1/22 (5%)	7/88 (8%)	0.35
Hepatitis*	22/110 (20%)	5/22 (23%)	3/88 (3%)	0,002*
Insulin-dependent Diabetes mellitus	12/110 (11%)	0/12 (0%)	8/98 (8%)	0,22

Supplementary Table 1

Association between clinical manifestations of APS-1 and the presence of KCNRG autoantibodies.

Fisher's two-tailed exact test was used to compare the data between the two groups of patients with autoantibodies directed against KCNRG, and the P values reported. A statistically significant correlation was found for pulmonary symptoms. Nevertheless, since the diagnosis of pulmonary symptoms is based on patient self-report, the entire cohort has not been subject to lung function test. Hence, the possible existence of unreported cases within the cohort should be taken in consideration when interpreting the statistical data. * It is notable that the presence of KCNRG autoantibodies appears to show a statistically significant correlation to hepatitis. The expression of KCNRG in hepatocellular carcinoma has previously been reported (Cho YG et al. Exp Mol Med. 2006;38(3):247-55). These findings could be in parallel, nevertheless, the sensitivity of the test appears to be higher for respiratory symptoms than hepatitis. Moreover, other well established and clinically reliable autoantigens for APS-1 associated hepatitis (Cytochrome P4501A2 and aromatic L-amino acid decarboxylase) are previously described and the autoantibodies directed against these autoantigens appear to have higher sensitivity and specificity for hepatitis, and hence, better markers than KCNRG for diagnosis of hepatitis.

Legends to Supplementary Figures

Supplementary Figure 1

Expression Analysis of KCNRG mRNA by Northern Blot.

Human multiple tissue Northern blot hybridized with ³²P-labeled KCNRG cDNA probe. Positions of molecular weight markers are shown on the left. The position of the ~1.35 kb band in the lung lane is indicated by the arrow.

Supplementary Figure 2

Sedimentation Analysis of ³⁵S-labeled *in vitro*-Translated Bovine KCNRG by Ultracentrifugation on a 10-40 % Sucrose Gradient. The ³⁵S-radiolabeled KCNRG was detected in collected fractions by autoradiography after separation by SDS-PAGE. The band volumes detected by Phosphor Imager and analyzed by the ImageQuaNT software (Molecular Dynamics), are plotted in the line graph. Positions of sedimentation standards are indicated by the vertical arrows. **B).** Size exclusion chromatography of ³⁵S-labeled bovine KCNRG synthesized by *in vitro* transcription and translation. Fractions were collected and the translation product was detected by scintillation counting. V₀, Void volume; V_t, total volume.

Supplementary Figure 3

Immunoprecipitation of ³⁵S-Radiolabelled KCNRG Isoforms with APS-1 Patient Sera.

Radiolabeled bovine KCNRG, and the 26kDa and 31kDa splice variants of human KCNRG, with sera from APS-1 patients with KCNRG autoantibodies (first three lanes from left), sera from APS-1 patients without KCNRG autoantibodies (lanes 4-6 from left), and sera from healthy blood donors (NHS) (lanes 7-8 from left). The immunoprecipitates were size determined by SDS-PAGE followed by autoradiography. These results indicate that the three different isoforms of KCNRG are recognized identically by sera from APS-1 patients.

Supplementary Figure 4

Immunoblot Experiment to Verify the Specificity of the KCNRG Antiserum

To test the specificity of the KCNRG antiserum, *in vitro*-translated bovine KCNRG (lane 1) and bovine lung tissue protein lysate (lanes 2-4) were separated by SDS-PAGE and transferred to a membrane. The membrane was blotted with the KCNRG antiserum in dilution 1:5000 for lane 1-2 and 1: 10000 for lane 3. In lane 4, the antiserum in dilution 1:10 000 was first preincubated overnight with 100 nmol of the peptide used for immunization of the rabbits. The antiserum recognizes *in vitro*-translated bovine KCNRG as well as KCNRG in the lung protein lysate.

Supplementary Figure 5

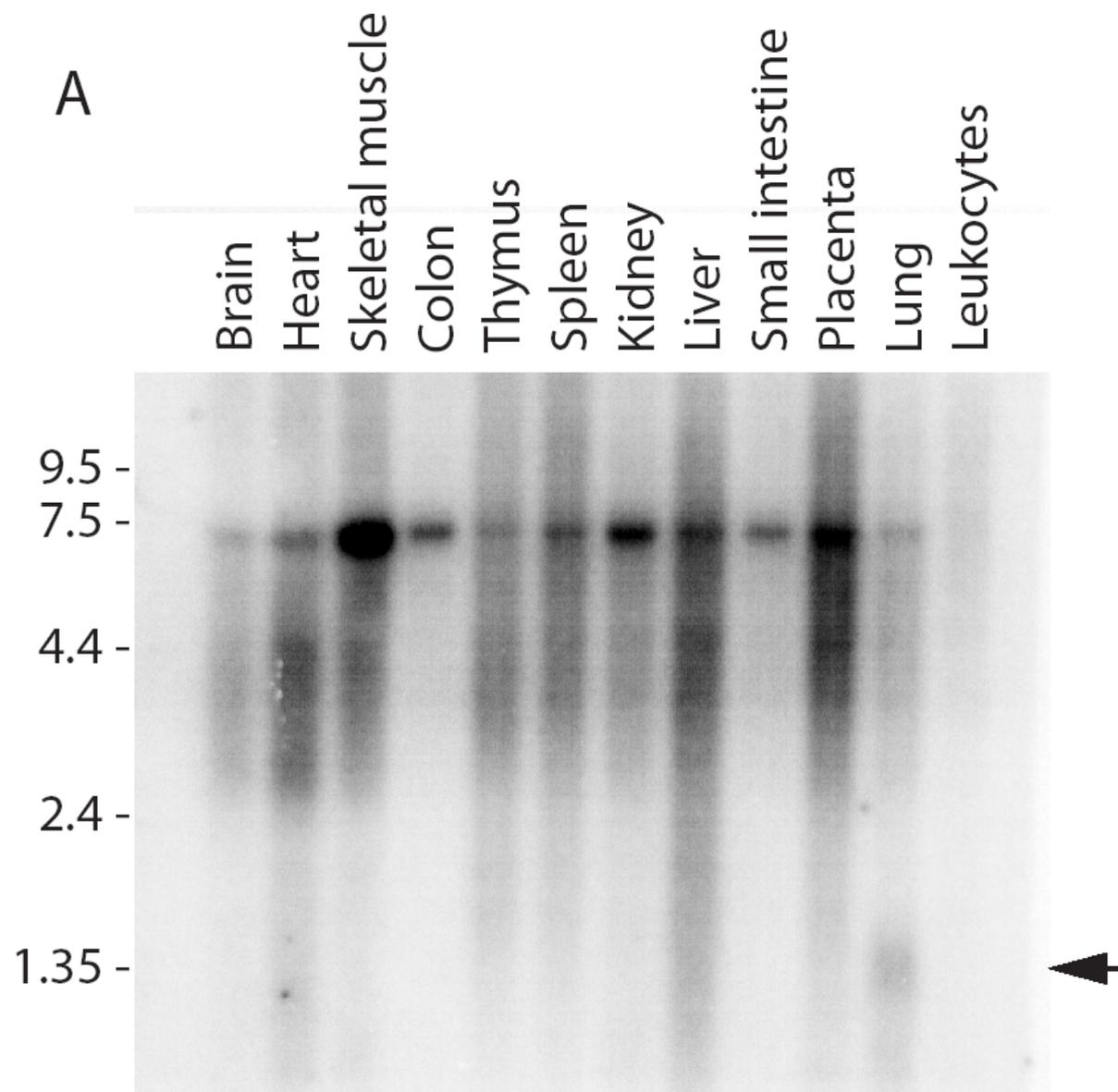
Preabsorption Studies to Confirm the Specificity of Autoantibodies for KCNRG in Patients with APS-1.

Immunofluorescence was performed on cryosections of bovine lung with the use of serum from two different APS-1 patients (patients 6 and 7 from Table 1). Panels A-C correspond to patient 6 and panels D-F correspond to patient 7. The serum samples were diluted (1:800) and preincubated with either no protein (panels A and D), or an equal amount of *in vitro* transcribed and translated KCNRG labeled with ³⁵S-methionine (panels B and E) or luciferase (Panels C and F). The results demonstrate that patient autoantibodies can be blocked by KCNRG and not by an irrelevant protein, suggesting that the autoantibodies directed to the terminal bronchioles are specific for KCNRG. Scale bars represent 100µm.

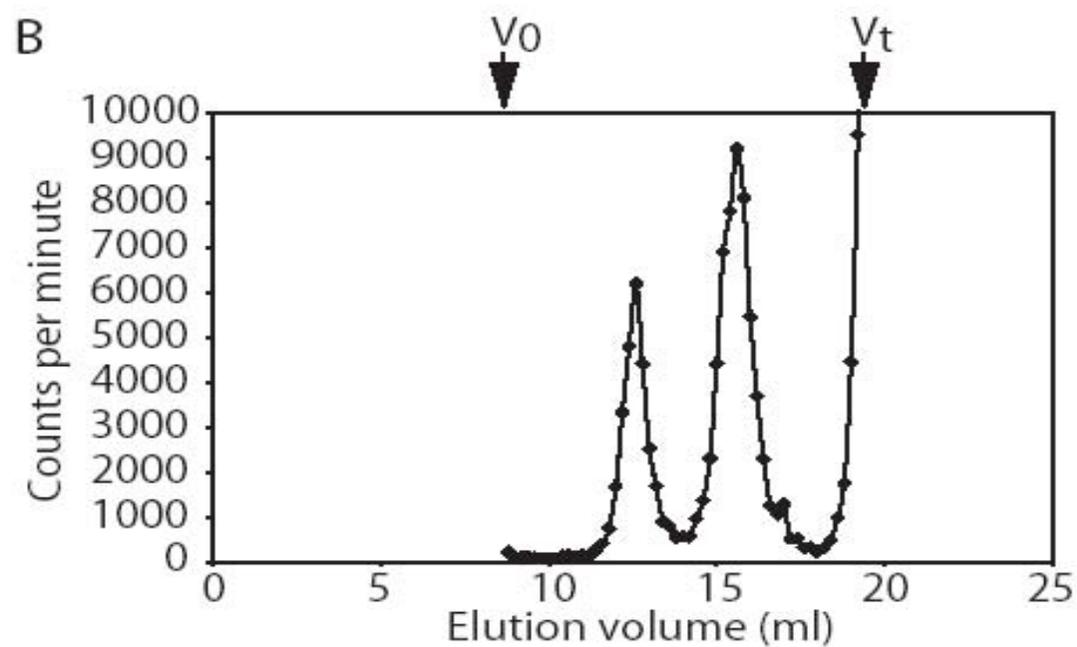
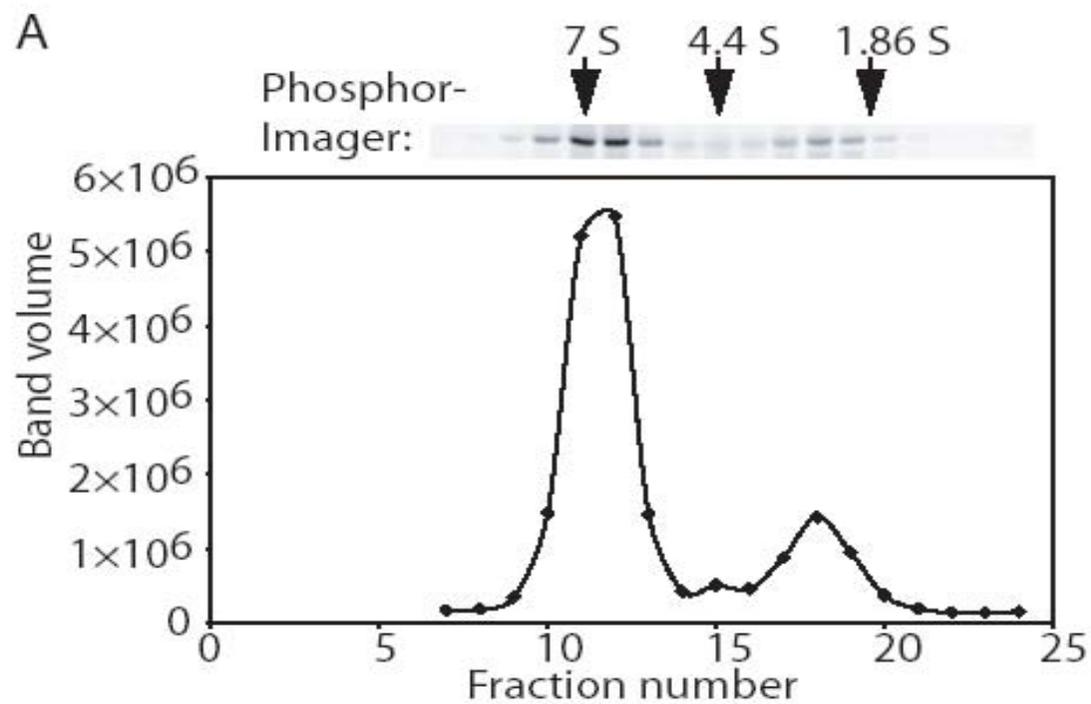
Supplementary Figure 6

Tissue Distribution of the KCNRG Protein. To exclude ubiquitous expression of KCNRG, the KCNRG antiserum was used in dilution 1:2000 to stain a human multi-tissue array previously described in the Swedish Human Protein Atlas Project (www.proteinatlas.org). The antiserum showed strong but unattractive staining of the tissue spot designated bronchus. Moreover weak staining could be seen in prostate and pancreas. Taken together, the tissue protein expression of KCNRG appears to be in line with the mRNA expression data presented in Figure 2B of the manuscript.

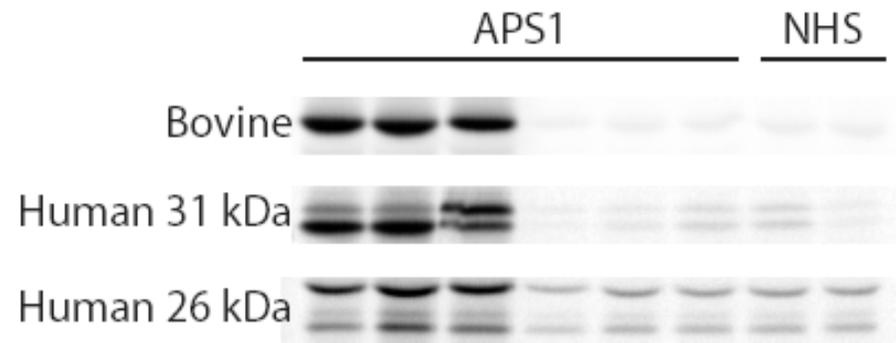
Supplementary Figure 1



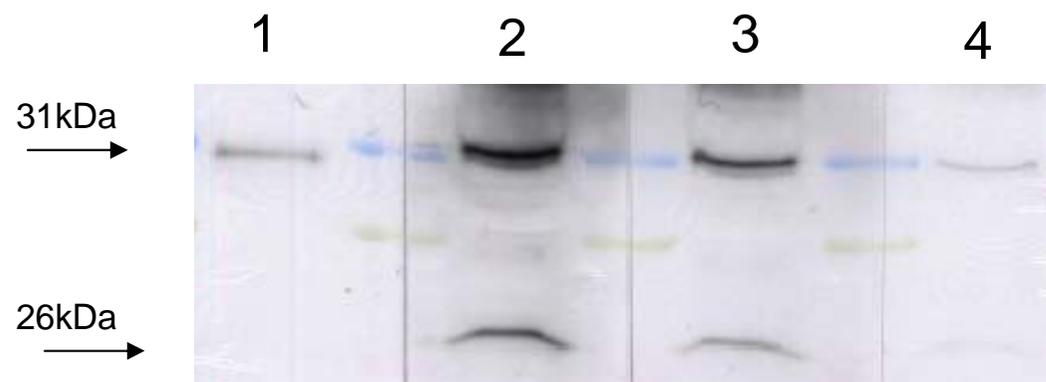
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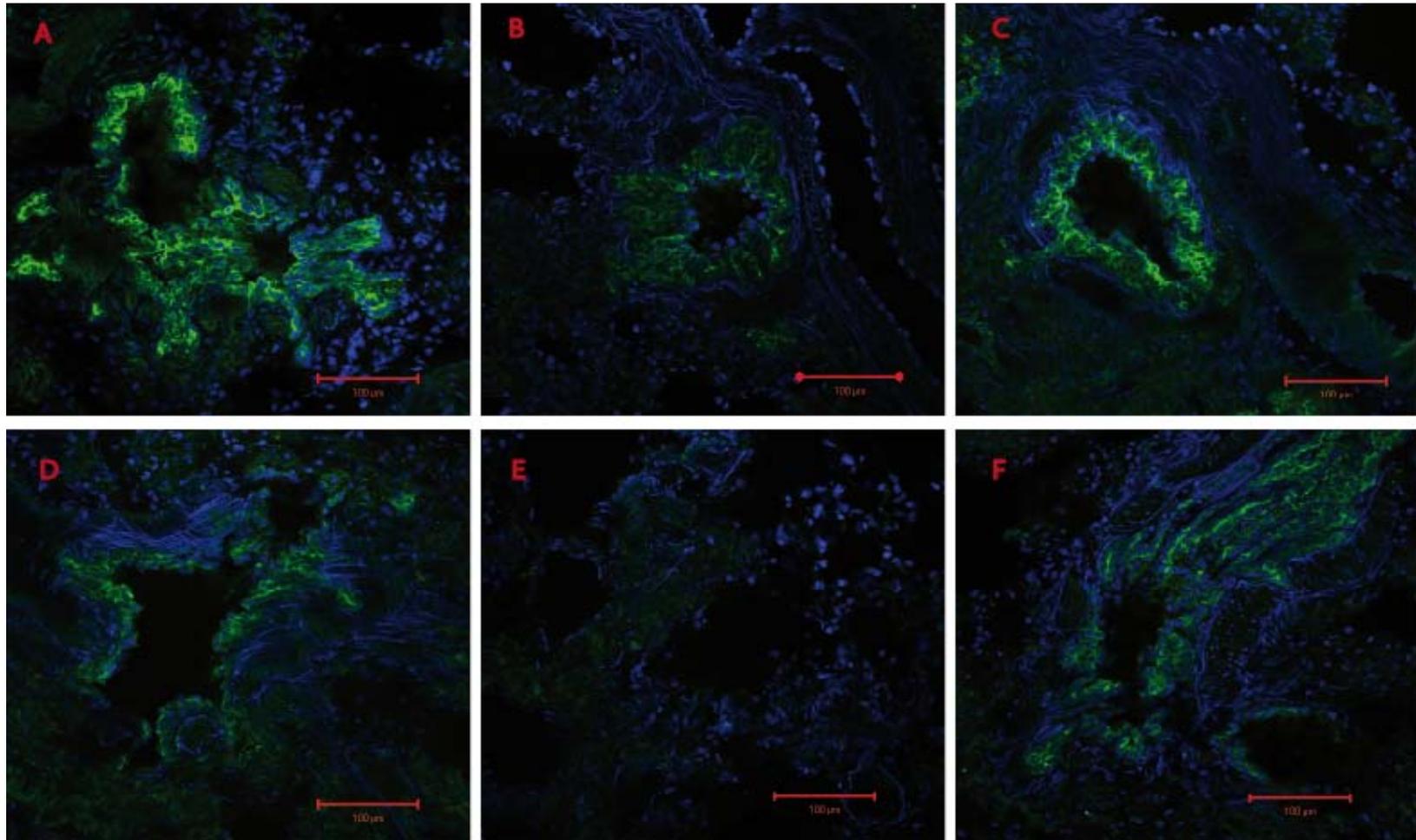
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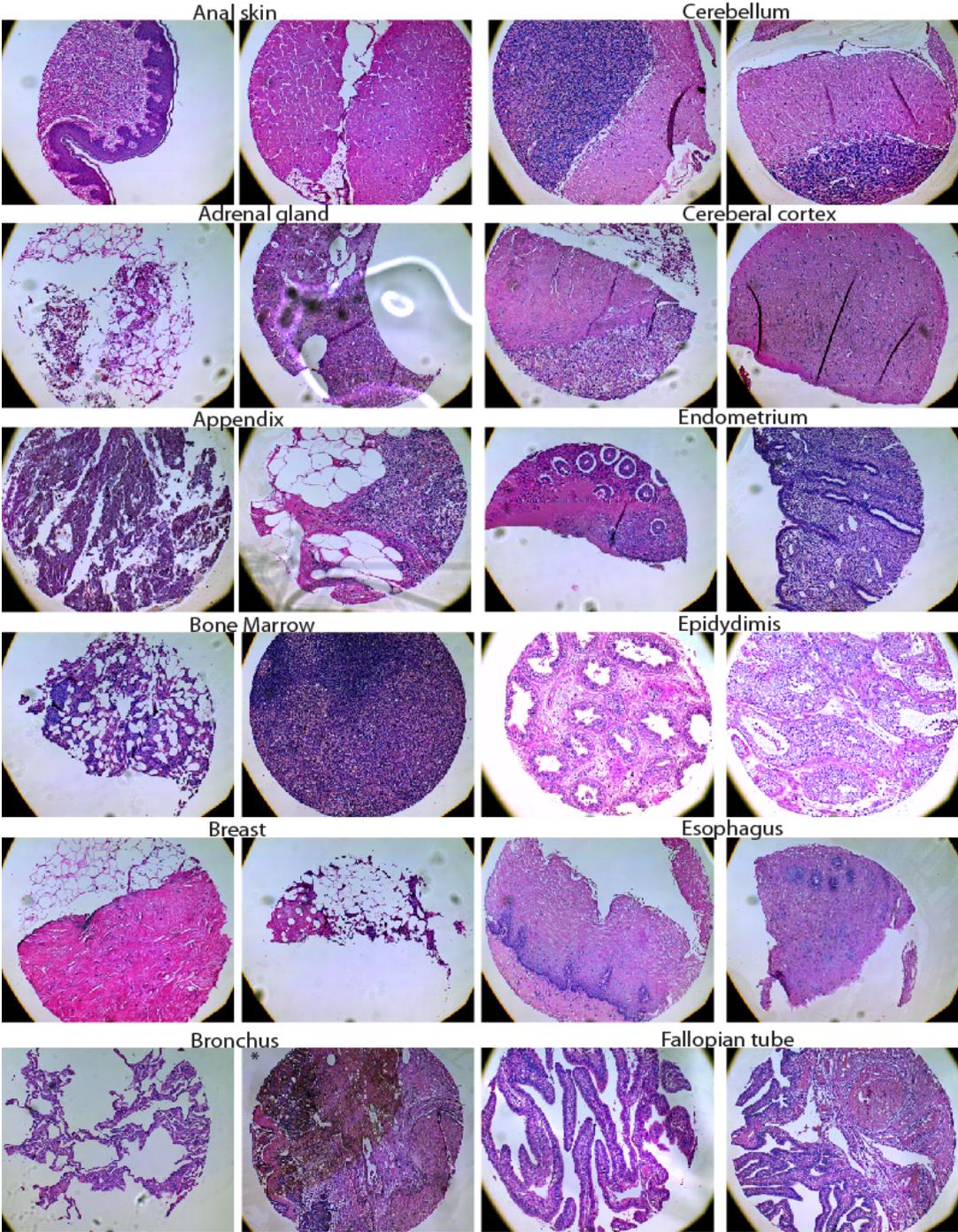
Supplementary Figure 4



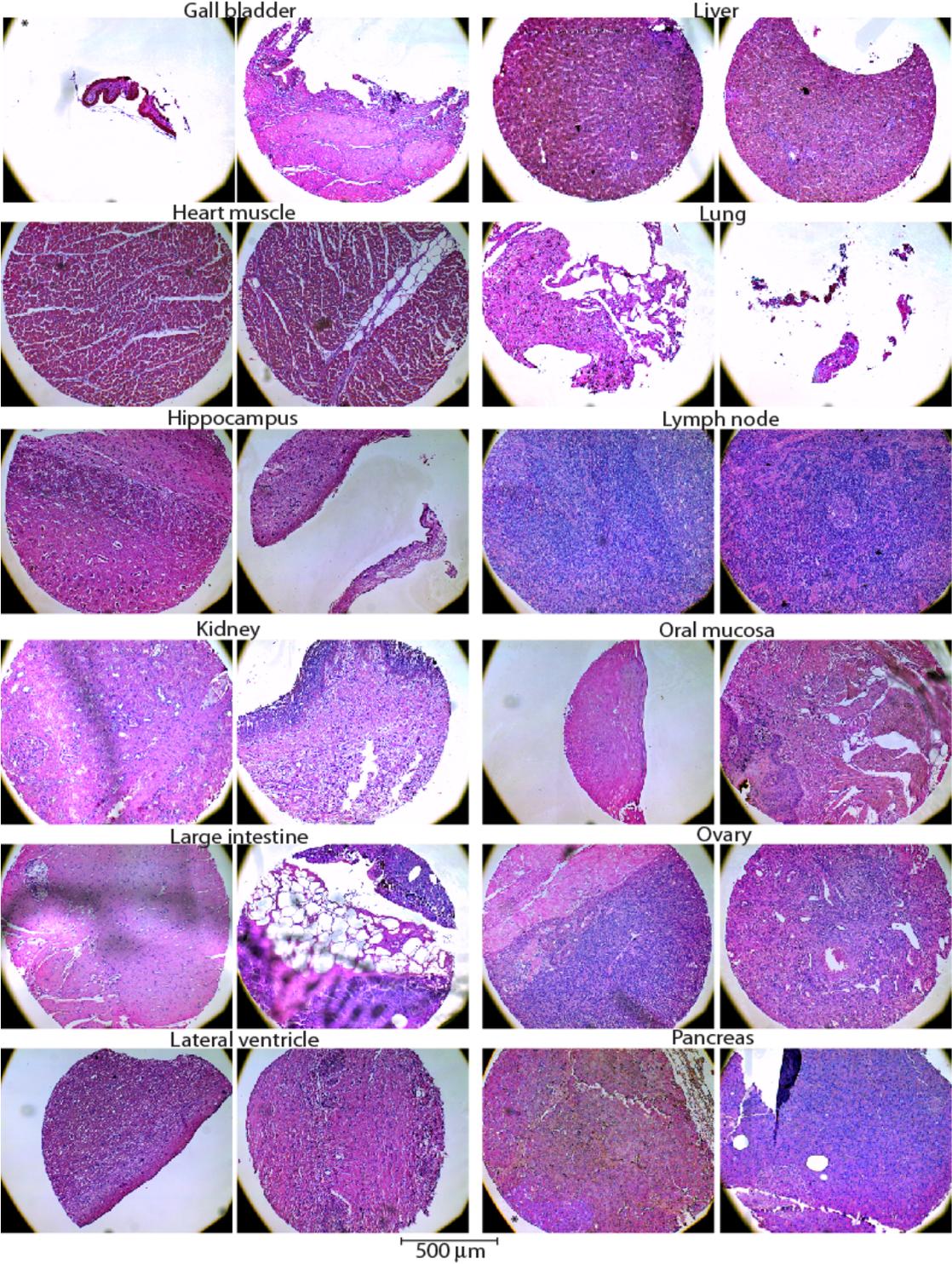
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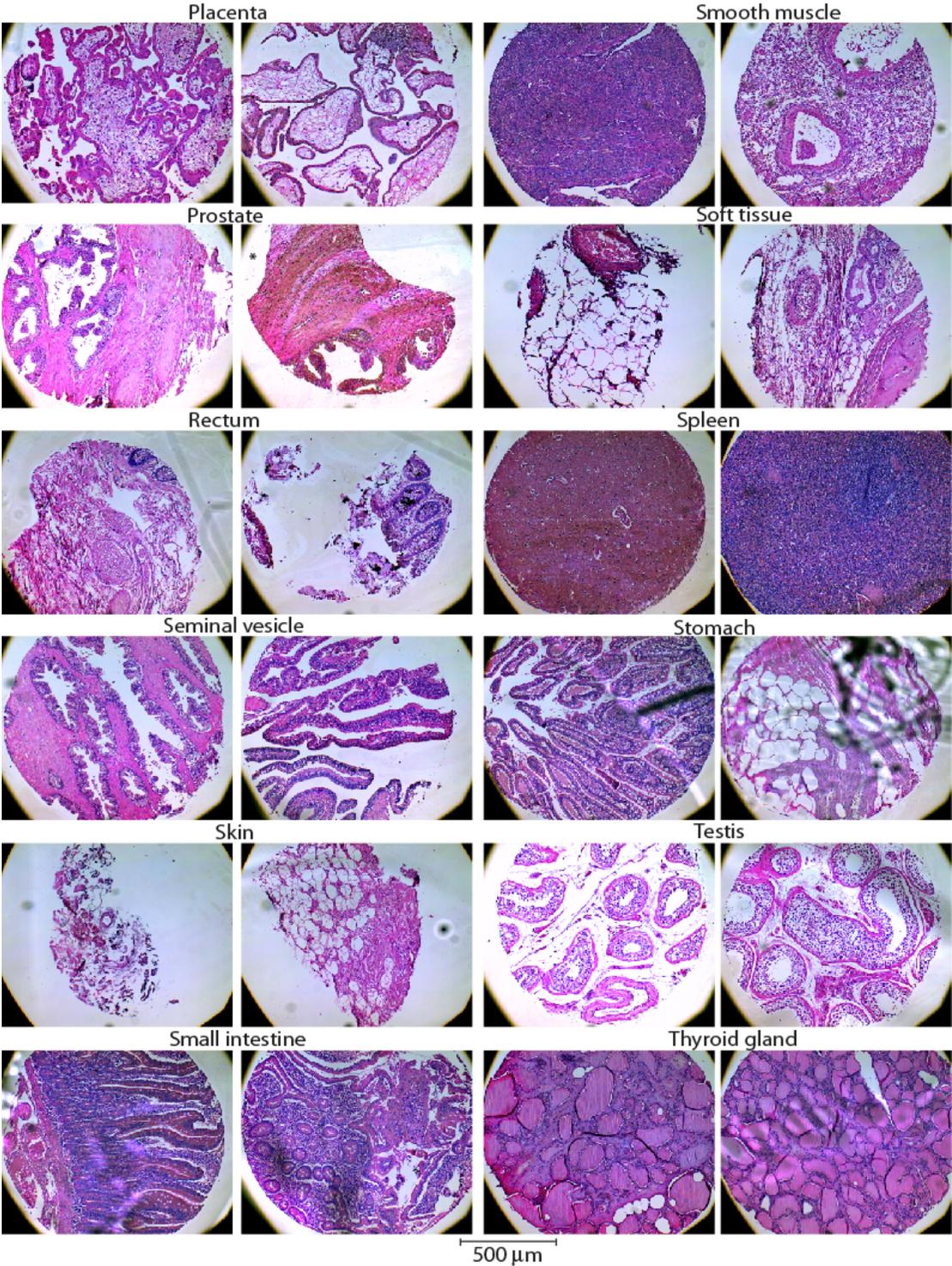
Supplementary Figure 6 Part 1



Supplementary Figure 6 Part 2



Supplementary Figure 6 Part 3



Supplementary Figure 6 Part 4

