

**Overexpression of Squamous Cell Carcinoma Antigen in Idiopathic Pulmonary Fibrosis:
clinico-pathological correlations.**

Fiorella Calabrese¹, Francesca Lunardi¹, Cinzia Giacometti¹, Giuseppe Marulli², Marianna Gnoato³, Patrizia Pontisso³, Marina Saetta², Marialuisa Valente¹, Federico Rea², Egle Perissinotto⁴, Carlo Agostini³.

¹Department of Diagnostic Medical Sciences and Special Therapies, University of Padua Medical School; Padua, Italy.

²Department of Cardiothoracic Sciences, University of Padua Medical School; Padua, Italy.

³Department of Clinical and Experimental Medicine, University of Padua Medical School; Padua, Italy.

⁴Department of Environmental Medicine and Public Health, University of Padua Medical School; Padua, Italy.

Corresponding Author:

Fiorella Calabrese, MD

Department of Diagnostic Medical Sciences and Special Therapies,
University of Padua Medical School.

Via Gabelli, 61. Padua, Italy.

Tel. +39049-8272268; Fax. +39049-8272269; e-mail: fiorella.calabrese@unipd.it

Key words: Idiopathic Pulmonary Fibrosis, SCCA, serpin, lung cancer.

Word count (body of the manuscript): 3713

ABSTRACT

Background Idiopathic pulmonary fibrosis (IPF) is a chronic progressive disorder with a poor prognosis. Epithelial instability is a crucial step in the development and progression of the disease including neoplastic transformation. Few tissue markers regarding epithelial instability have been reported in IPF. Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor typically expressed by dysplastic and neoplastic cells of epithelial origin, more often in squamous cell tumours. At present no information is available on its expression in IPF.

Methods SCCA and transforming growth factor- β (TGF- β) expression in surgical lung biopsies from 22 IPF patients and 20 control cases. An in vitro study using A549 pneumocytes was also conducted to investigate the relationship between SCCA and TGF β expression. SCCA and TGF β epithelial expression were evaluated by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). SCCA values were correlated with different pathological and clinical parameters. Time course analysis of TGF- β expression in A549 pneumocytes incubated with different SCCA concentrations was assessed by real time RT-PCR.

Results SCCA was expressed in many metaplastic alveolar epithelial cells in all IPF cases with a mean value of 24.9% while it was seen in only two control patients in up to 5% of metaplastic cells. In IPF patients SCCA correlated positively with the extension of fibroblastic foci ($r = 0.49$, $p = 0.02$), the expression of TGF- β ($r = 0.78$, $p < 0.0001$) and with DLCO decline at 9 months of follow-up ($r = 0.59$, $p = 0.01$). In vitro experiments showed that incubation of cultured cells with SCCA induced TGF- β expression, with a peak at 24 hours.

Conclusion Our findings provide for the first time a potential mechanism by which SCCA secreted from metaplastic epithelial cells may exert a profibrotic effect in IPF. SCCA could be an important biomarker in this incurable disease.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), morphologically characterized by an usual interstitial pneumonia (UIP), represents a progressive disease of unknown aetiology that continues to be associated with high morbidity and mortality [1].

The key pathological features of this disorder include epithelial damage/activation, fibroblastic/myofibroblastic foci formation and extracellular matrix remodelling [2]. Although previous studies on fibrogenesis have focused on the role of inflammation in favouring fibroblast activation and fibrosis, the current paradigm suggests the pivotal role of epithelial deregulation in the disease pathogenesis [2-3]. Epithelial injury and subsequent deregulated repair result in cytokine release, excess of extracellular matrix deposition, and abnormal mesenchymal cell activation and proliferation. Among profibrogenetic cytokines, transforming growth factor- β (TGF- β) represents the key growth factor which leads to mesenchymal differentiation, myofibroblast infiltration and proliferation. As a consequence the areas of immature fibrosis are progressively invaded by small aggregates of actively proliferating myofibroblasts and fibroblasts termed fibroblastic foci (FF) whose number and extension have been associated with the prognosis of the disease [4].

Epithelial instability has been largely documented by the presence of frequent evidence of morphologic changes through variegated cellular alterations such as hyperplasia, atypia, squamous metaplasia, dysplasia and eventually carcinoma [5-9]. The frequent occurrence of lung carcinoma, particularly squamous cell type, in patients with IPF/UIP is one of the contributing factors to a poor prognosis [10]. A few tissue markers regarding epithelial instability have recently been described to be over expressed in lung tissue from patients with IPF/UIP including the expression of the K-ras gene with point mutation and the presence of multiple mutations of p53 which have been detected in type II alveolar pneumocytes of IPF/UIP lungs [11]. Also an overexpression of wild type p53 has been reported by several authors. Its upregulation in the face of chronic DNA damage may increase the potential p53 mutation leading to an imbalance of different growth factors and consequently increased tumorigenesis in IPF [12-13]. A more frequent occurrence of guanine nitration has recently been found in metaplastic and neoplastic cells from patients with IPF/UIP indicating that nitrative stress is an important step in epithelial damage at risk of neoplastic transformation [14].

Squamous cell carcinoma antigen (SCCA) is a serine protease inhibitor typically expressed by dysplastic/neoplastic cells of epithelial origin, including lung cancer [15]. Recent studies indicate that both SCCA1 and SCCA2, the two isoforms so far identified [16], protect neoplastic cells from apoptotic death induced by several kinds of stimuli, and in vivo experiments demonstrate that SCCA can promote tumor growth [17]. The main goal of the present study was to assess the expression of SCCA both as mRNA and protein in lung biopsies from IPF/UIP and control patients. To analyse the pathogenetic and prognostic value of SCCA, the association between its value and different clinical and pathological data including TGF- β tissue expression was studied. An in vitro study was also conducted to investigate biological activity of SCCA on TGF- β expression.

MATERIALS AND METHODS

Patients

The study was performed on lung samples from 22 patients with IPF consecutively evaluated from June 2003 to August 2006 in our Centre and on 20 samples from non-IPF subjects. The diagnosis of IPF was based on the diagnostic criteria of the American Thoracic Society/European Respiratory Society Consensus Classification System [18]. Samples from IPF patients were obtained from video-assisted thoracoscopic lung biopsies. Written informed consent was obtained from each patient and the work was approved by the Institutional Ethics Committee. Histological examination revealed all the major features of UIP, which is a prerequisite for the diagnosis of IPF. The mean age of the patients was 60.2 years (range 44 to 69 years); 17 of the patients were males and five females. All patients underwent routine pulmonary function testing, including spirometry, lung volume measurement, measurement of diffusion capacity of carbon monoxide (DLCO), arterial blood gases at rest and after exercise, chest radiography and high resolution computed tomography (HRCT). The main characteristics of studied subjects are shown in Table 1. Lung function data were recorded less than six weeks before biopsies in all IPF cases and re-evaluated after a median period of 9 months (range 6-11 months). At follow-up, clinical data were completed for 18 patients, two patients died before undergoing the second pulmonary function test and spirometry results were not evaluable for two cases. Data were expressed as percentage of values predicted from the subject's age, sex and height. Fifteen patients underwent biopsies at two different sites (upper and lower lobes), and the other seven patients were biopsied at three separate sites (upper, middle and lower lobes), giving a total number of 51 biopsies. The majority of the patients were treated after biopsy with a high dose of steroids alone or associated with azathyoprine. Control lungs were obtained from non implanted donor lungs (10 cases) and from other forms of interstitial lung diseases (ILDs, 10 cases): two non-specific interstitial pneumonias (NSIP), one desquamative interstitial pneumonia (DIP), one Langerhans-cell histiocytosis (LCH), one lymphangioleiomyomatosis (LAM), two respiratory bronchiolitis interstitial lung diseases (RBILD), one cryptogenic organizing pneumonia (COP), one mixed pneumoconiosis and one hypersensitivity pneumonitis (HP). The donors (5 males and 5 females, mean age 30 ± 17 years, all no smokers) died of cerebral trauma and stayed less than two days in intensive care without evidence of lung infections or other complications. Patients affected by other forms of ILDs (3 males and 7 females; mean age: 45 ± 15 years, all smokers except LAM and HP patients) were defined by the presence of clinical, radiological and histological evidence of specific ILD. All lung tissues were formalin-fixed and paraffin-embedded following standard protocols.

Table 1: Clinical and pathological characteristics of the study population (22 UIP patients)

Characteristics	UIP patients	
		range
Sex (male:female)	17 : 5	
Status (dead/alive)	2 : 20	
Age at biopsy (years, mean \pm SD)	60.2 \pm 6.2	44 - 69
Dust exposure (yes/no)	9 : 13	
Smoker (yes/no)	12 : 10	
Smoking (pack-years, mean \pm SD)	23.3 \pm 19.1	0.45 - 64
Follow-up (months, median)	9	6 - 11
<i>Pathological features</i>		
Fibroblastic foci score (%)	1	4.6
	2	40.9
	3	13.6
	4	22.7
	5	4.6
	6	13.6
Fibroblastic foci (A _{FF} %, median)	8.4	4.3 – 21.8
Fibrosis extension (A _{Fib} %, mean \pm SD)	35.6 \pm 7.4	22 – 51.8
Inflammation score (%)	1	53.8
	2	38.5
	3	7.7
Inflammation (A _{IC} %, mean \pm SD)	3.0 \pm 1.4	1.2 – 6.6
<i>Spirometry at time of diagnosis</i>		
DLCO (% predicted, mean \pm SD)	53.7 \pm 12.7	23 - 77
FEV1 (% predicted, mean \pm SD)	72.9 \pm 15.7	42 - 109
FVC (% predicted, mean \pm SD)	69.5 \pm 13.6	49 - 97
VC (% predicted, mean \pm SD)	71.1 \pm 12.7	47 - 94
TLC (% predicted, mean \pm SD)	67.1 \pm 12.9	48 - 91
RV (% predicted, mean \pm SD)	67.7 \pm 25.5	31 – 125

A_{FF}, fibroblastic foci area; A_{Fib}, fibrotic area; A_{IC}, inflammatory cells area; DLCO, Diffusing lung capacity for carbon monoxide; FEV1, forced expiratory volume in one second; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; VC, vital capacity. For normally distributed quantitative variables, mean \pm SD range are shown; for not normally distributed quantitative variables, median range and IQR are shown; for categorical variables percentage distributions are shown.

Histology and morphometry

Fibroblastic foci (FF) and inflammatory cells (IC) were evaluated by a semi quantitative method. In particular, FF were analyzed by using a Brompton score [4], and IC was scored as follows: less than 10% of lung tissue examined (score 1), more than 10% and less than 30% of lung tissue examined (score 2), more than 30% of lung tissue examined (score 3). In all samples from each patient, the extension of fibrosis, inflammation and FF were also measured by computerized morphometric analyses (Image Pro-plus version 5). The extension of fibrosis was quantified on lung sections stained by Azan-Mallory as previously described [19]. FF, IC and fibrosis were analyzed on ten random fields in the same section of imaged lesions at 50-fold magnification excluding the areas of honeycombing. In each selected field, the ratio of fibroblastic foci, inflammatory cells and fibrotic areas (A_{FF} , A_{IC} , A_{FIB}) were calculated dividing the total A_{FF} , A_{IC} and A_{FIB} by the total tissue area (excluding airspaces) of the section (where n = the number of fields): FF Ratio = $\sum_n A_{FF} / \text{Total Area} * 100$, IC Ratio = $\sum_n A_{IC} / \text{Total Area} * 100$, Fibrosis Ratio = $\sum_n A_{FIB} / \text{Total Area} * 100$. For each patient, the ratios obtained from the analysed sections were then averaged and this value was correlated with all pathological and clinical parameters. Differently from the authors recently supporting the value of quantitative analysis of FF [20], we measured all parameters including FF exclusively related to lung tissue (excluding air spaces) to normalize the effect of collapse or expansion of lung tissue during biopsy or tissue fixation.

Immunohistochemical analysis

All cases were immunoassayed with a novel polyclonal rabbit antibody anti-SCCA (Hepa-Ab, Xeptagen, Venice, Italy) and mouse monoclonal anti-TGF- β (NovoCastra, Newcastle, UK) as previously described [21,22]. All samples were processed using a sensitive avidin-streptavidin-peroxidase technique and stained with a mixture of 3,3-diamino-benzidine tetra hydrochloride and hydrogen peroxide. Parallel control slides were prepared either lacking primary antibody or lacking primary and secondary antibodies, or were stained with normal sera to control for background reactivity. Consecutive serial sections immunostained for SCCA and TGF- β were evaluated and the quantification was restricted to strongly stained metaplastic epithelial cells (cuboidal, squamous, bronchiolar) A total of 500 metaplastic epithelial cells for each patient were counted in remodelled lung parenchyma (at least two sections) and the value was expressed as a percentage of positive cells/500 for each case. This value was correlated with all pathological and clinical values.

Molecular analysis

Manual tissue dissection

Manual tissue dissection of representative areas, positive for SCCA and TGF- β , was performed in half of the cases (11 cases), in which metaplastic epithelial aggregates were easily dissected. Briefly, five sequential 5 μ m sections from formalin-fixed paraffin-embedded blocks were placed on non-coated glass slides and coupled with SCCA and TGF- β immunostained tissue sections. The areas (at least 1 mm in diameter) carefully marked to easily compare the unstained levels were gently scraped with a sterile scalpel. The procured tissue fragments were then placed in a tube, deparaffinized and washed in xylene and alcohol before nucleic acid extraction. After this procedure, the remaining unselected tissue was stained with hematoxylin-eosin to verify the isolated tissue parts.

Areas of normal tissue (negative for SCCA and TGF- β immunoassaying) from the same paraffin block and from donor lung were also dissected and processed in the same way.

RT-PCR of SCCA and TGF- β

Total RNA was extracted by using the modified RNAzol method, as previously described [23]. The RNA pellet was redissolved in 15 μ l sterile DEPC-treated water and incubated with 1 μ l of RNase inhibitor (Applied Biosystems, Milan, Italy) and 20 U of DNase I (Sigma Aldrich, Milan, Italy) for two hours at 37 C in a total volume of 20 μ l. The oligonucleotides used to ascertain the quality of extracted RNA were complementary to the mRNA glyceraldehyde-3-phosphate dehydrogenase

(GAPDH). The sequences of primers for GAPDH, SCCA and TGF- β , annealing temperature condition and amplicon sizes are listed in Table 2. At least 1 μ g of extracted total RNA was used for the first complementary DNA (cDNA) synthesis and conventional RT-PCR was used. The PCR mix was made up to a volume of 50 μ l using 1X PCR Buffer II, 1mM MgCl₂ solution, 200 μ M each of dATP, dCTP, dGTP, dUTP, 400 nM of each primer, and 1.25 Units of AmpliTaq Gold. After the initial denaturation at 95°C for 10 min, the cDNA was amplified by 40 three-step cycles (30 sec at 95°C, 30 sec at annealing temperature, 1 min at 72°C). SCCA and TGF- β amplicons were both verified by previously described gene sequencing protocol [21].

Table 2. Oligonucleotide sequences of primers used to amplify GAPDH (housekeeping), TGF- β and SCCA.

Primer	Sequence 5' - 3'	Annealing Temperature	Amplicon size
GAPDH Fw	GGGCTCTCCAGAACATCATCC	60	130 bp
GAPDH Rv	GTCCACCACTGACACGTTGG		
SCCA Fw	GGCAGCTGCAGCTTCTG	55	80 bp
SCCA Rv	AGCCGCGGTCTCGTGC		
TGF- β Fw	GCCCTGGACACCAACTATTGC	60	161 bp
TGF- β Rv	AGGCTCCAAATGTAGGGGCAG		

Bp, base pair; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Fw, forward; Rv, reverse; SCCA, squamous cell carcinoma antigen; TGF- β , transforming growth factor- β .

In vitro study

Cell culture – Lung epithelial cell line A549 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained routinely in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (Gibco, Milan, Italy) and supplemented with 100 mg/ml penicillin G (Gibco, Milan, Italy) and 100 mg/ml streptomycin sulfate (Gibco, Milan, Italy). Cells were then seeded in DMEM with 10% fetal bovine serum at a density of 0.3×10^6 cells per well in 6-well plates. All the experiments were performed at 100% cell confluence. Time course analysis was carried out to assess the effect of SCCA on TGF- β expression. Cells were incubated with SCCA (Xeptagen, Venice, Italy) at increasing concentrations (range: 1-1000 pg/ml) and checked for TGF- β expression at 6-hour intervals (range: 6-48 hours). Cells were collected and lysated immediately with RLT buffer (Qiagen, Milan, Italy) and β -Mercaptoethanol 14.5 M 1% (Sigma Aldrich, Milan, Italy).

Quantitative RT-PCR of TGF- β

RNA was extracted using RNeasy Mini Kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. RNA was reverse-transcribed using the Reverse Transcription System (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Real Time PCR was performed using a standard TaqMan[®] PCR kit protocol on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Milan, Italy). The TaqMan[®] PCR was carried out in a 96-well microtiter plate format (Applied Biosystems). The PCR mix was made up to a volume of 25 μ l using ready-to-use Universal Mastermix containing AmpliTaq DNA polymerase, uracil-N-glycosylase (UNG), dNTPs, KCl, MgCl₂ and ROX as passive reference all in optimized concentrations. After UNG treatment at 50° C for 2 min and initial denaturation at 95°C for 10 min, the DNA was amplified by 40 two-step cycles (15 sec at 95°C, 1 min at 57°C). All reactions were run in triplicate. Reactions and cycling were performed as recommended by the manufacturer's instructions. GAPDH was used as a reference gene for the adjustment of relative expression data. Naive cells collected at the same time were used as calibrators. All assays were

performed in triplicate to ensure their reproducibility, and a negative control was included in each run. Primers and probe for GAPDH and TGF- β were commercially available (4333764 and 4327054, Applied Biosystems).

Statistical analysis

Normality of distribution for quantitative variables was assessed by means of Shapiro-Wilk statistics. Normally distributed quantitative variables are described as mean value and standard deviation, not normal quantitative variables are expressed as median, range and IQR, while categorical variables are presented as percentage distribution. To evaluate simple linear relationships between quantitative variables, Pearson's or Spearman's correlation coefficients were applied, as necessary. To evaluate the independent association of each factor with the dependent variable, the partial correlation coefficients were estimated.

In the analysis of the relationship between SCCA and scores, to allow greater set sizes, foci and inflammation scores were used to group IPF subjects. For foci, the subjects ranked: 1 (score 1 to 2), 2 (score 3 to 4) or 3 (score 5 to 6). For inflammation, the variable was dichotomized (score ≤ 1 , score > 1). The mean value of SCCA of the different groups was compared by means of one-way analysis of variance (ANOVA).

Statistical analysis was performed using SAS statistical software version 9.1 (SAS Institute, Cary, NC, USA). P values lower than 0.05 were considered statistically significant.

RESULTS

Pathological findings and clinico-pathological correlations

Median histological score (range, IQR) was 3.0 (1 to 6, 2) and 1.0 (1 to 3, 1) for FF and for IC, respectively. Among all the quantitative variables only fibroblasti foci area (A_{FF}) showed a distribution different from normality ($p = 0.02$). Median value of fibroblastic foci area (A_{FF}) was 8.4% (4.3% to 21.8%, 5.6%). Mean value of inflammation area (A_{IC}) and fibrosis extension were $3.0 \pm 1.4\%$ and $35.6 \pm 7.4\%$, respectively (Table 1). A direct correlation was observed between A_{FF} and A_{FIB} ($r = 0.56$, $p = 0.007$). A statistically significant correlation was observed only between A_{FIB} and decline of DLCO at nine months ($r = 0.5156$, $p = 0.0302$).

Immunohistochemical findings and correlations with morphological and clinical data

SCCA was expressed in many metaplastic alveolar epithelial cells in all UIP cases. It varied in the range 9.4 to 44.0% and was normally distributed with mean $24.9 \pm 9.3\%$ (Fig. 1A, 1B). Immunoassaying was mainly detected in the cytoplasm however in a few cases cytoplasmic and nuclear staining was also observed (Fig. 2A). Cuboidal, flattened metaplastic epithelial cells showed a wide spectrum of staining from strong to weak SCCA positivity (Fig. 2B) in contrast to squamous and bronchiolar metaplastic epithelial cells which frequently showed a strong staining (Fig. 2C), more often well evident in or close to the honeycomb changes. Interstitial cells were negative for SCCA expression and a weak cytoplasmic staining was observed in some alveolar macrophages. Metaplastic epithelial SCCA positive staining was seen in only two ILDs different from IPF (NSIP and DIP). The positivity was weak and mainly detected in cytoplasmic areas with a mean of $0.4 \pm 1.3\%$ (from 0 to 5%). The pseudostratified columnar epithelial cells lining the bronchi and bronchioles often exhibited strong cytoplasmic immunoreactivity for SCCA, thus representing a good internal immunostaining control. No alveolar cells were detected in normal lung tissue from donor subjects when only some bronchial cells were marked (Fig. 3).

In all UIP cases TGF- β , which often marked the same metaplastic epithelial cells positively stained for SCCA, showed a mean value of $29.8 \pm 13.5\%$ (7.1 to 55.6%) and was significantly correlated with the expression values of SCCA ($r = 0.78$, $p < 0.0001$) (Fig 4A, B, C). TGF- β epithelial expression was occasionally seen in control group (up to 2%).

A direct correlation was observed between the expression of SCCA and A_{FF} ($r = 0.49$, $p = 0.02$) (Fig 5A) and between TGF- β and A_{FF} ($r = 0.44$, $p = 0.04$). Controlling for SCCA values, the correlation between TGF- β and A_{FF} was no longer statistically significant (partial $r = 0.10$, $p = 0.67$). Mean SCCA values did not significantly differ both among foci score groups ($F = 0.65$, $p = 0.53$) and inflammation score groups ($F = 0.36$, $p = 0.56$). The only statistically significant relationship between SCCA values and lung clinical data variation during follow-up was observed with decreasing DLCO (Δ DLCO) at nine months ($r = 0.38$, $p = 0.04$) (Fig 5B).

SCCA and TGF- β mRNAs

The expected 80 and 161 base pair PCR products of SCCA and TGF- β , respectively, were detected from all pathological micro dissected lung tissues, confirming epithelial transcription (data not shown).

TGF- β mRNA expression in A549 cells incubated with SCCA

Time course analysis of the effect of SCCA on TGF- β transcription in A549 cell lines is reported in Figure 6. The peak of induction of TGF- β transcription was achieved at 24 hours, with the highest activity of SCCA at the 100 pg/ml concentration.

DISCUSSION

The serpin superfamily includes inhibitors of a number of serine proteases with roles in a variety of cellular processes, including fibrinolysis, inflammation, cell migration, adhesion and proliferation. SCCA is transcribed from the tandemly repeated genes, SCCA1 and SCCA2, which have 98% sequence identity at the nucleotide level and 92% identity at the amino-acid level [16]. The products of the two genes have different protease targets; SCCA1 inhibits papain-like cysteine proteases (cathepsin S, L and K) [24] and SCCA 2 chymotrypsin-like serine proteases (e.g. cathepsin G and mast cell chymase) [25]. While some studies have reported increased SCCA expression (mainly in blood serum) in many tumors, particularly those with squamous cell differentiation [26-27], and in preneoplastic bronchial lesions [28], little is known about the behaviour of this serpin in a non-neoplastic clinical setting.

In the present study we have demonstrated for the first time the overexpression of SCCA in lung tissue of IPF/UIP patients compared to other forms of ILDs and normal lungs. In IPF, SCCA was abnormally secreted by metaplastic epithelial cells other than bronchial cells where it is normally expressed. The normal presence of SCCA at this level, as was demonstrated in our control cases, could have a protective function against inflammatory cells and micro organisms because SCCA 1 and 2 are inhibitors of serine and cysteine proteinase. Previous studies have reported that SCCA can have an important influence on epithelial growth through inhibition of different apoptotic pathways [29, 30]. The marked positivity of SCCA in the honeycomb area suggests that this site is more frequently subjected to injury favoring epithelial proliferation, which could be highly unstable when recurrent injuries occur.

Several years ago Meyer and Liebow already showed atypical epithelial lesions in honeycombing pulmonary areas in IPF raising the possibility that atypical epithelial lesions in IPF might be precancerous lesions [5]. Squamous metaplasia in our samples showed a frequent and strong staining with SCCA. It is probable that this epithelial setting represents a regeneration epithelial site of high instability at risk of neoplastic transformation. A recent *in vitro* study has shown an over-expression of SCCA in squamous metaplastic tracheo-bronchial cell lines, increasing with tumor progression [31]. Squamous type carcinoma has in fact been detected in IPF/UIP lungs more frequently than other tumour types [9].

The nuclear/cytoplasmic concomitant SCCA immunoreactivity observed in our samples is difficult to interpret. Other works have recently described nuclear-cytoplasmic positive staining of serpin (maspin and SCCA) in non small cell lung carcinoma. In this work the nuclear maspin positivity was correlated with a better survival than with cytoplasmic staining [32]. The synthesis of SCCA is supposed to be essentially in the cytoplasm. However when it is overexpressed, nuclear import might occur thus playing a role in influencing the transcription of different growth factor genes such as TGF- β . A significant overexpression of TGF- β was detected in our cases and interestingly it was significantly correlated with SCCA expression. The increased TGF- β transcription by SCCA observed in A549 pneumocytes confirms clinical observations detected in patients with IPF/UIP. Unfortunately, it is nearly impossible to work with primary alveolar epithelial cells, and we had to resort to the epithelial cell line A549, employed in numerous studies of IPF. There are different sources of TGF- β in IPF/UIP lungs, and active TGF- β transcription was detected in many metaplastic epithelial cells obtained by micro dissection of our samples. Active epithelial secretion of this profibrogenetic cytokine was first reported by Corrin *et al.* [33] and subsequently by other authors [34], thus underlining the important contributing role of epithelial cells in the development/progression of fibrotic process in IPF/UIP.

The significant correlation between the expression of SCCA and the extension of FF and fibrosis could be mediated through the increased transcription of the fibrotic cytokine TGF- β , confirming the role of this cytokine as one of the most important inducers of fibrotic processes in IPF/UIP. Our morphological evaluation confirms the prognostic significance of the quantitative measure of fibrosis and FF (instead of semi quantitative evaluation) in that these are the only pathological findings significantly correlated with DLCO decline and SCCA expression, as above reported. The

significant correlation of SCCA with DLCO decline observed in our patients at nine months of follow-up could be related to progressive fibrosis favoured by increased TGF- β transcription. However it remains to be established if alveolar epithelial dysfunction could represent an important contributing factor for progressive impaired gas exchange.

Although our findings need to be confirmed in larger case series we consider SCCA an important molecular target in the disease as it could orchestrate two of the most peculiar aspects of the disease: this serpin could act in an autocrine way favouring epithelial proliferation and metaplastic dysplastic transformation while in a paracrine way it could influence miofibroblast proliferation and collagen synthesis through increased TGF- β transcription (Fig 7). Monitoring of tissue SCCA expression during the clinical course of IPF/UIP could be useful for more precise patient stratification. It could be advisable to use a non-invasive approach such as that innovative serological analysis of SCCA-immunoglobulin M complex, a more sensitive and appropriate technique [35] that those previously used.

A final issue that needs to be taken into account is the possible role of SCCA in the development of lung cancer in IPF/UIP. The analysis of this specific aspect could be proven in studies considering tissue samples of cancer in UIP and by using specific experimental models as those regarding transgenic SCCA animal models recently engineered in our lab.

ACKNOWLEDGMENTS

The authors thank Luca Braghetto and Laura Vignato for their excellent technical assistance and Judith Wilson for English revision.

FUNDING

This study was supported by the Italian Ministry of Instruction, University and Research.

COMPETING INTERESTS

All the authors have no relationship that may pose any conflict of interest.

COPYRIGHT

The corresponding author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its Licensees to permit this article to be published in Thorax editions and any other BMJPG products to exploit all subsidiary rights, as set out in our licence <http://thorax.bmjournals.com/ifora/licence.pdf>.

LEGENDS

Fig. 1 Immunohistochemistry for SCCA.

A: UIP case, female, 56 years old (SCCA mean value: 44%). Many metaplastic bronchiolar and cuboidal cells are strongly marked. Original magnification X 50.

B: UIP case, male, 65 years old (SCCA mean value: 10.5%). A few metaplastic cuboidal and bronchiolar cells are moderately stained. Original magnification X 50.

Fig. 2 Immunohistochemistry for SCCA.

UIP case, male, 66 years old (SCCA mean value: 32%). Strong nuclear and cytoplasmic staining of metaplastic bronchiolar cells (**A**), weak cytoplasmic staining of metaplastic cuboidal cells (**B**), both original magnification X 350, and strong cytoplasmic and nuclear staining of metaplastic squamous cells (**C**), original magnification X 250.

Fig. 3 Immunohistochemistry for SCCA.

Control case (non implanted donor lung): positive staining is seen only in epithelial cells of bronchial tract with well developed BALT. Original magnification X 50.

Fig. 4 Immunohistochemistry for SCCA and TGF- β (A, B); SCCA and TGF- β correlation (C).

UIP case, female, 61 years old. Note the strong staining in the same metaplastic cells of sequential serial sections, original magnification X 300 (**A** and **B**). Significant correlation between SCCA and TGF- β epithelial expression ($r = 0.78$, $p < 0.0001$) (**C**).

Fig. 5 SCCA- A_{FF} correlation (A); SCCA- Δ DLCO correlation (B)

Direct correlation was observed between A_{FF} and SCCA expression ($r = 0.49$, $p = 0.02$) (**A**). Significant correlation was seen between SCCA expression and Δ DLCO ($r = 0.38$, $p = 0.04$) (**B**).

Fig. 6 Effect of SCCA at different time points in A549 cell line.

At 24 hours a peak of TGF- β RNA transcription was observed and the highest induction was achieved with 100 pg/ml SCCA concentration.

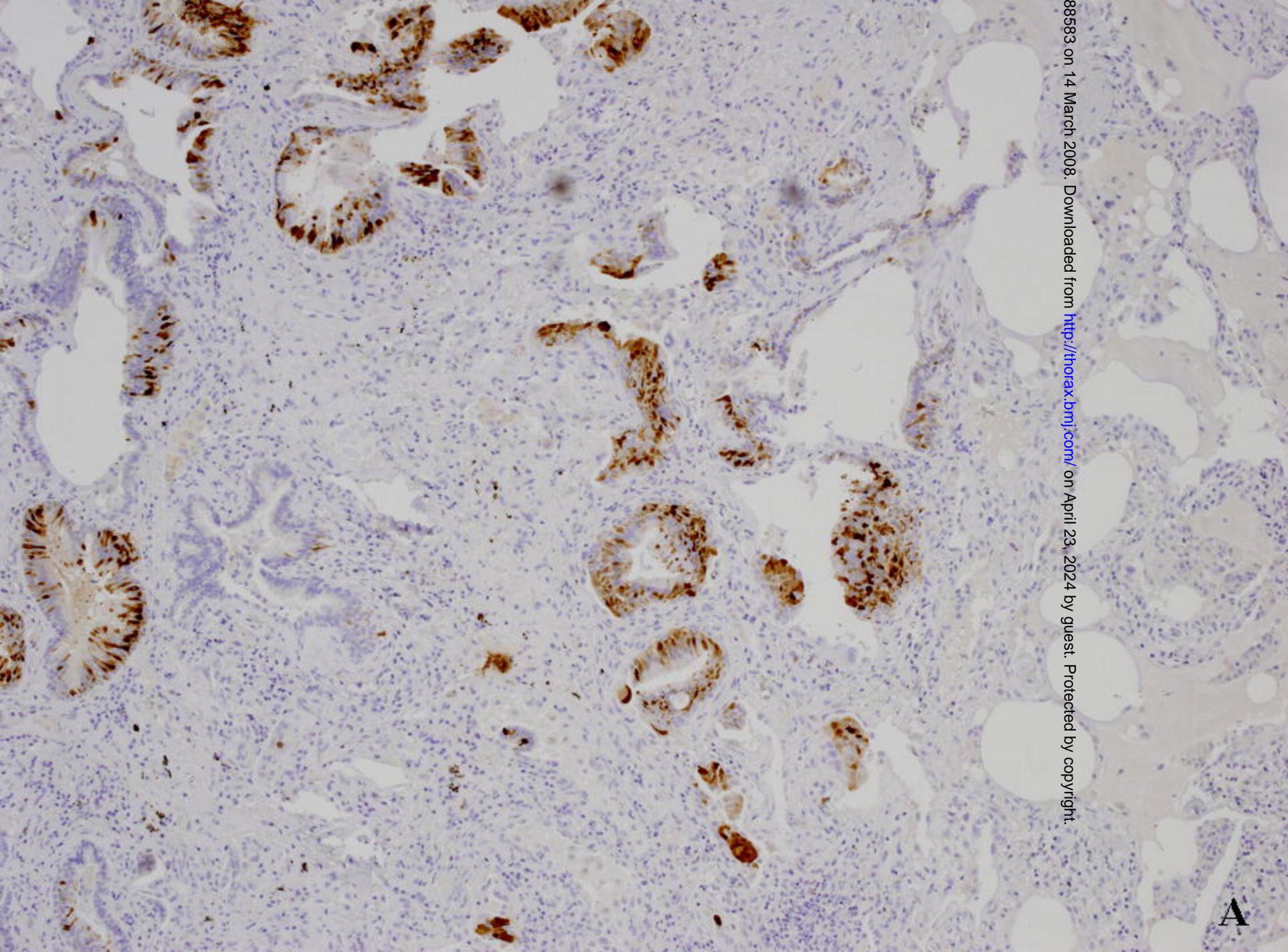
Fig. 7 Novel putative schema showing SCCA pathway in UIP

SCCA could play a crucial role in the development of the disease: influencing epithelial proliferation (autocrine action) or promoting fibroblast proliferation/fibrosis through increased TGF- β secretion (paracrine action).

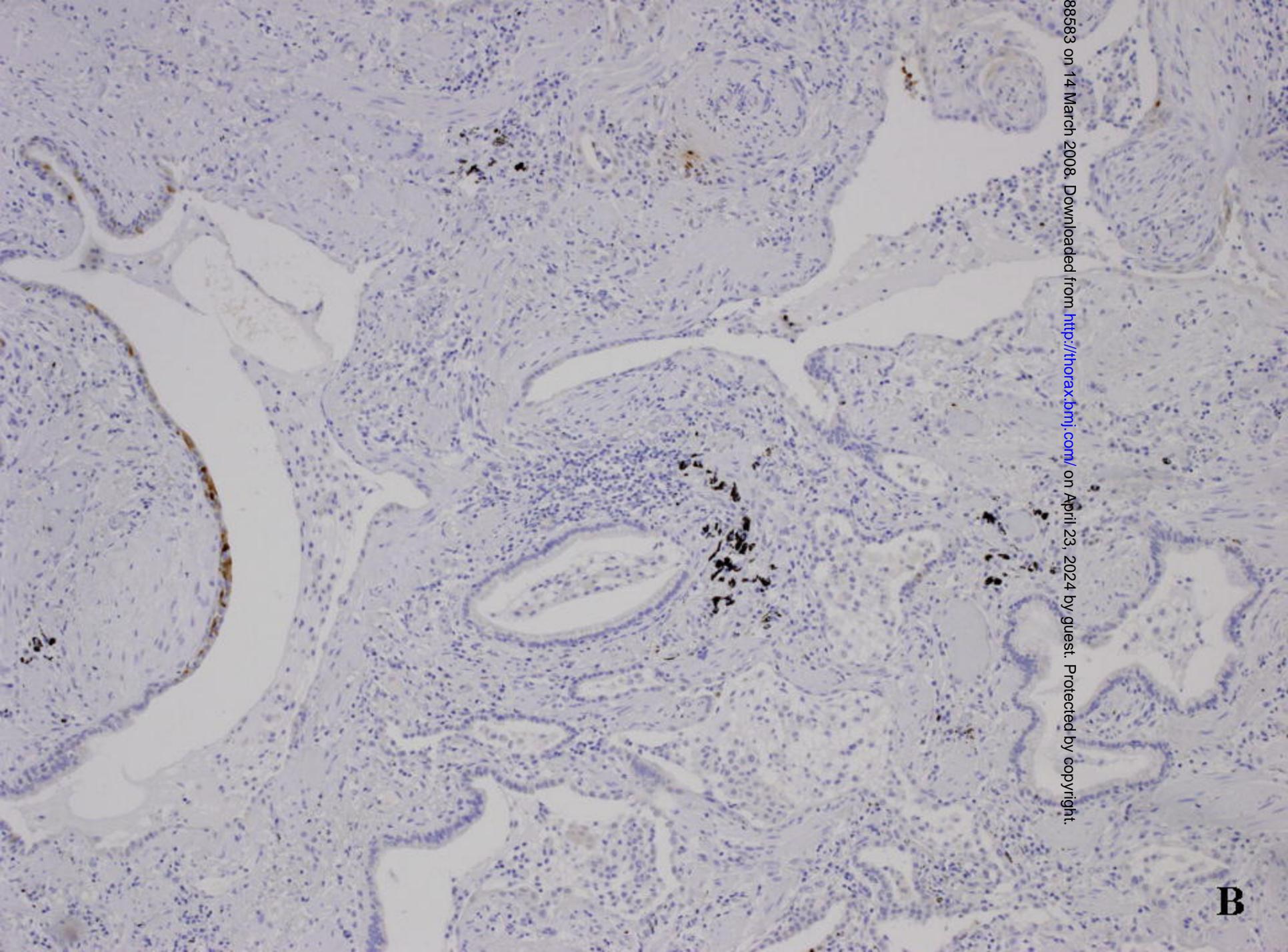
REFERENCES

1. Crystal RG, Bitterman PB, Mossman B, *et al.* Future research directions in idiopathic pulmonary fibrosis: summary of a National Heart, Lung, and Blood Institute working group. *Am J Respir Crit Care Med* 2002; **166**:236-46.
2. Selman M, King TE, Pardo A; American Thoracic Society; European Respiratory Society; American College of Chest Physicians. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001; **134**:136-51.
3. Calabrese F, Giacometti C, Rea F, *et al.* Idiopathic interstitial pneumonias: Primum movens: epithelial, endothelial or whatever. *Sarcoidosis Vasc Diffuse Lung Dis* 2005; **22**:S15-23.
4. Nicholson AG, Fulford LG, Colby TV, *et al.* The relationship between individual histologic features and disease progression in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2002; **166**:173-77.
5. Meyer EC, Liebow AA. Relationship of interstitial pneumonia honeycombing and atypical epithelial proliferation to cancer of the lung. *Cancer* 1965; **18**:322-51.
6. Hironaka M, Fukayama M. Pulmonary fibrosis and lung carcinoma: a comparative study of metaplastic epithelia in honeycombed areas of usual interstitial pneumonia with or without lung carcinoma. *Pathol Int* 1999; **49**:1060-66.
7. Hubbard R, Venn A, Lewis S, *et al.* Lung cancer and cryptogenic fibrosing alveolitis. A population-based cohort study. *Am J Respir Crit Care Med* 2000; **161**:5-8.
8. Park J, Kim DS, Shim TS, *et al.* Lung cancer in patients with idiopathic pulmonary fibrosis. *Eur Respir J* 2001; **17**:1216-19.
9. Aubry MC, Myers JL, Douglas WW, *et al.* Primary pulmonary carcinoma in patients with idiopathic pulmonary fibrosis. *Mayo Clin Proc* 2002; **77**:763-70.
10. Ma Y, Seneviratne CK, Koss M. Idiopathic pulmonary fibrosis and malignancy. *Curr Opin Pulm Med* 2001; **7**:278-82.
11. Takahashi T, Munakata M, Ohtsuka Y, *et al.* Expression and alteration of ras and p53 proteins in patients with lung carcinoma accompanied by idiopathic pulmonary fibrosis. *Cancer* 2002; **95**:624-33.
12. Kuwano K, Kunitake R, Kawasaki M, *et al.* P21Waf1/Cip1/Sdi1 and p53 expression in association with DNA strand breaks in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1996; **154**:477-83.
13. Chilosi M, Poletti V, Murer B, *et al.* Abnormal re-epithelialization and lung remodeling in idiopathic pulmonary fibrosis: the role of deltaN-p63. *Lab Invest* 2002; **82**:1335-45.
14. Terasaki Y, Akuta T, Terasaki M, *et al.* Guanine nitration in idiopathic pulmonary fibrosis and its implication for carcinogenesis. *Am J Respir Crit Care Med* 2006; **174**:665-73.
15. Kato H. Expression and function of squamous cell carcinoma antigen. *Anticancer Res* 1996; **16**:2149-53.
16. Schneider SS, Schick C, Fish KE, *et al.* A serine proteinase inhibitor locus at 18q21.3 contains a tandem duplication of the human squamous cell carcinoma antigen gene. *Proc Natl Acad Sci U S A* 1995; **92**:3147-51.
17. Suminami Y, Nagashima S, Murakami A, *et al.* Suppression of a squamous cell carcinoma (SCC)-related serpin, SCC antigen, inhibits tumor growth with increased intratumor infiltration of natural killer cells. *Cancer Res* 2001; **61**:1776-80.
18. American Thoracic Society; European Respiratory Society. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. *Am J Respir Crit Care Med* 2002; **165**:277-304.

19. Dona M, Dell'Aica I, Pezzato E, *et al.* Hyperforin inhibits cancer invasion and metastasis. *Cancer Res* 2004; **64**:6225-32.
20. Enomoto N, Suda T, Kato M, *et al.* Quantitative analysis of fibroblastic foci in usual interstitial pneumonia. *Chest* 2006; **130**:22-9.
21. Pontisso P, Calabrese F, Benvegna L, *et al.* Overexpression of squamous cell carcinoma antigen variants in hepatocellular carcinoma. *Br J Cancer* 2004; **90**:833-37.
22. Beghe B, Bazzan E, Baraldo S, *et al.* Transforming growth factor-beta type II receptor in pulmonary arteries of patients with very severe COPD. *Eur Respir J* 2006; **28**:556-62.
23. Chomczynski P, Sacchi N. Single-step method of RNA isolation by guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**:156-59
24. Schick C, Pemberton PA, Shi GP, *et al.* Cross-class inhibition of the cysteine proteinases cathepsins K, L, and S by the serpin squamous cell carcinoma antigen 1: a kinetic analysis. *Biochemistry* 1998; **37**:5258-66.
25. Schick C, Kamachi Y, Bartuski AJ, *et al.* Squamous cell carcinoma antigen 2 is a novel serpin that inhibits the chymotrypsin-like proteinases cathepsin G and mast cell chymase. *J Biol Chem* 1997; **272**:1849-55.
26. Body JJ, Sculier JP, Raymakers N, *et al.* Evaluation of squamous cell carcinoma antigen as a new marker for lung cancer. *Cancer* 1990; **65**:1552-56.
27. Vassilakopoulos T, Troupis T, Sotiropoulou C, *et al.* Diagnostic and prognostic significance of squamous cell carcinoma antigen in non-small cell lung cancer. *Lung Cancer* 2001; **32**:137-44.
28. Smith SL, Watson SG, Ratschiller D, *et al.* Maspin - the most commonly-expressed gene of the 18q21.3 serpin cluster in lung cancer - is strongly expressed in preneoplastic bronchial lesions. *Oncogene* 2003; **22**:8677-87.
29. Murakami A, Suminami Y, Hirakawa H, *et al.* Squamous cell carcinoma antigen suppresses radiation-induced cell death. *Br J Cancer* 2001; **84**:851-58.
30. Suminami Y, Kishi F, Sekiguchi K, *et al.* Squamous cell carcinoma antigen is a new member of the serine protease inhibitors. *Biochem Biophys Res Commun* 1991; **181**:51-8.
31. Lonardo F, Li X, Siddiq F, *et al.* Maspin nuclear localization is linked to favorable morphological features in pulmonary adenocarcinoma. *Lung Cancer* 2006; **51**:31-9.
32. Kim SW, Cheon K, Kim CH, *et al.* Proteomics-based identification of proteins secreted in apical surface fluid of squamous metaplastic human tracheobronchial epithelial cells cultured by three-dimensional organotypic air-liquid interface method. *Cancer Res* 2007; **67**:6565-73.
33. Corrin B, Butcher D, McAnulty BJ, *et al.* Immunohistochemical localization of transforming growth factor-beta 1 in the lungs of patients with systemic sclerosis, cryptogenic fibrosing alveolitis and other lung disorders. *Histopathology* 1994; **24**:145-50.
34. Khalil N, O'Connor RN, Flanders KC, *et al.* TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am J Respir Cell Mol Biol* 1996; **14**:131-38.
35. Beneduce L, Castaldi F, Marino M, *et al.* Squamous cell carcinoma antigen-immunoglobulin M complexes as novel biomarkers for hepatocellular carcinoma. *Cancer* 2005; **103**:2558-65.

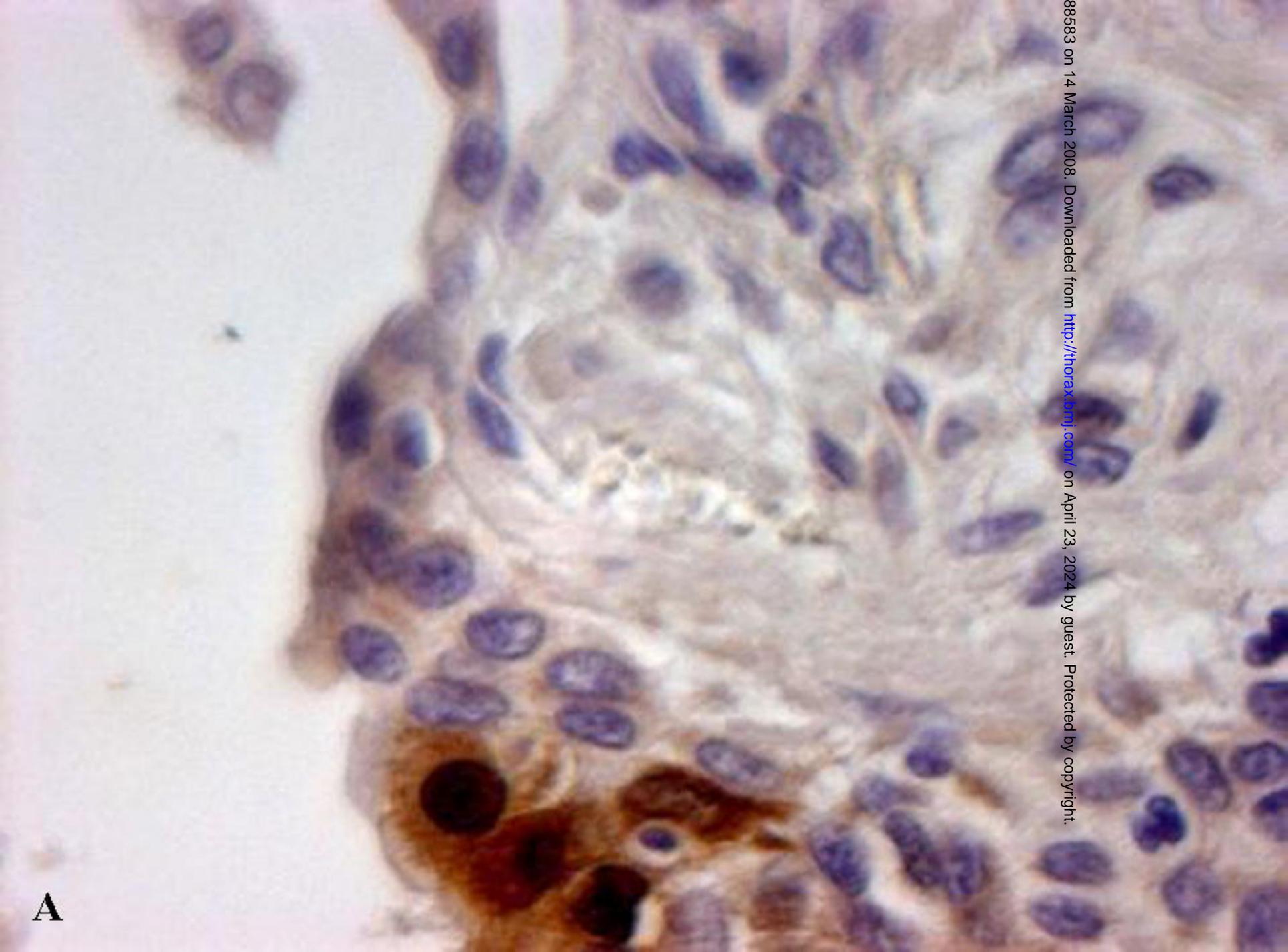


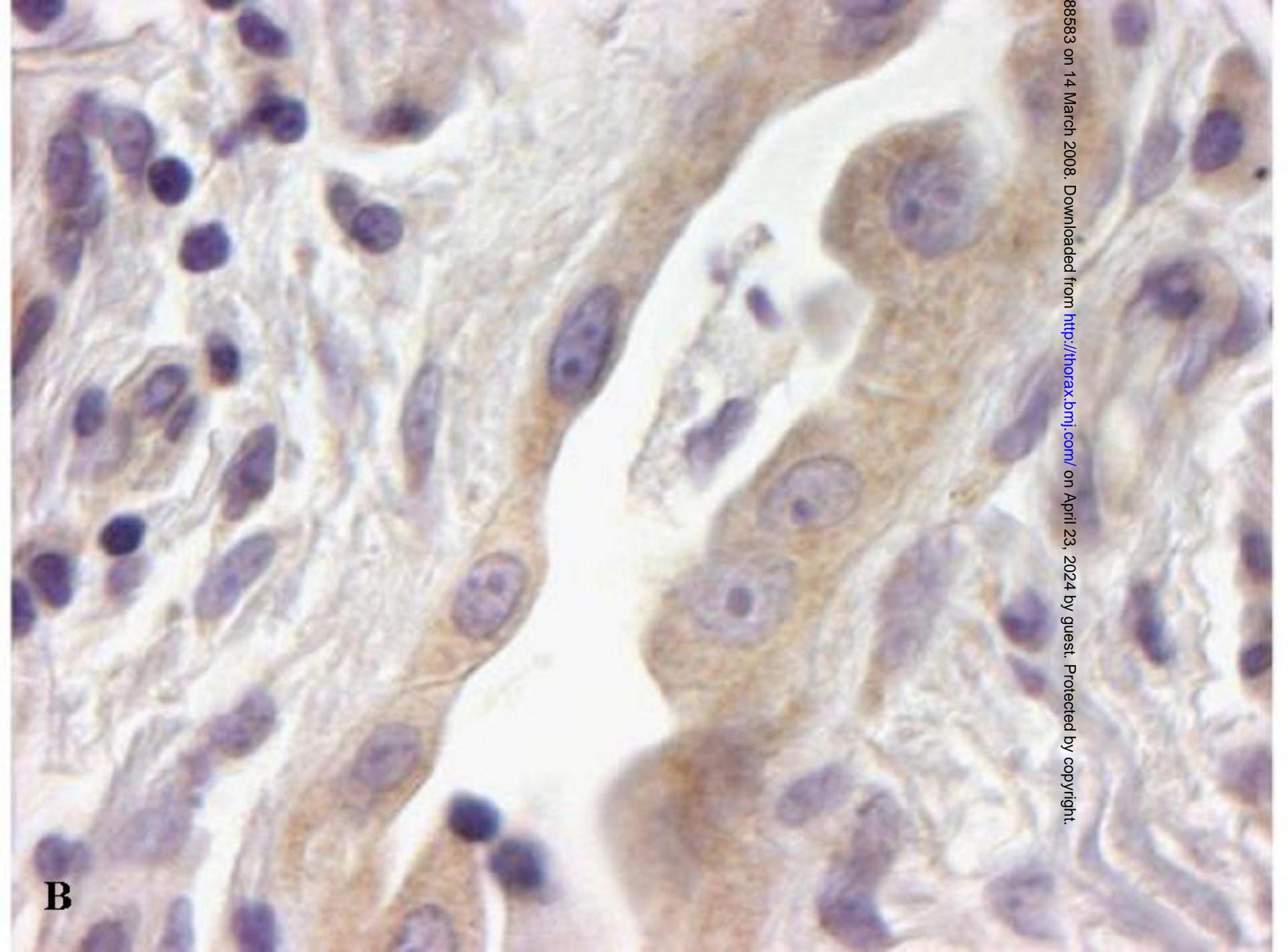
886863 on 14 March 2008. Downloaded from <http://thorax.bmj.com/> on April 23, 2024 by guest. Protected by copyright.



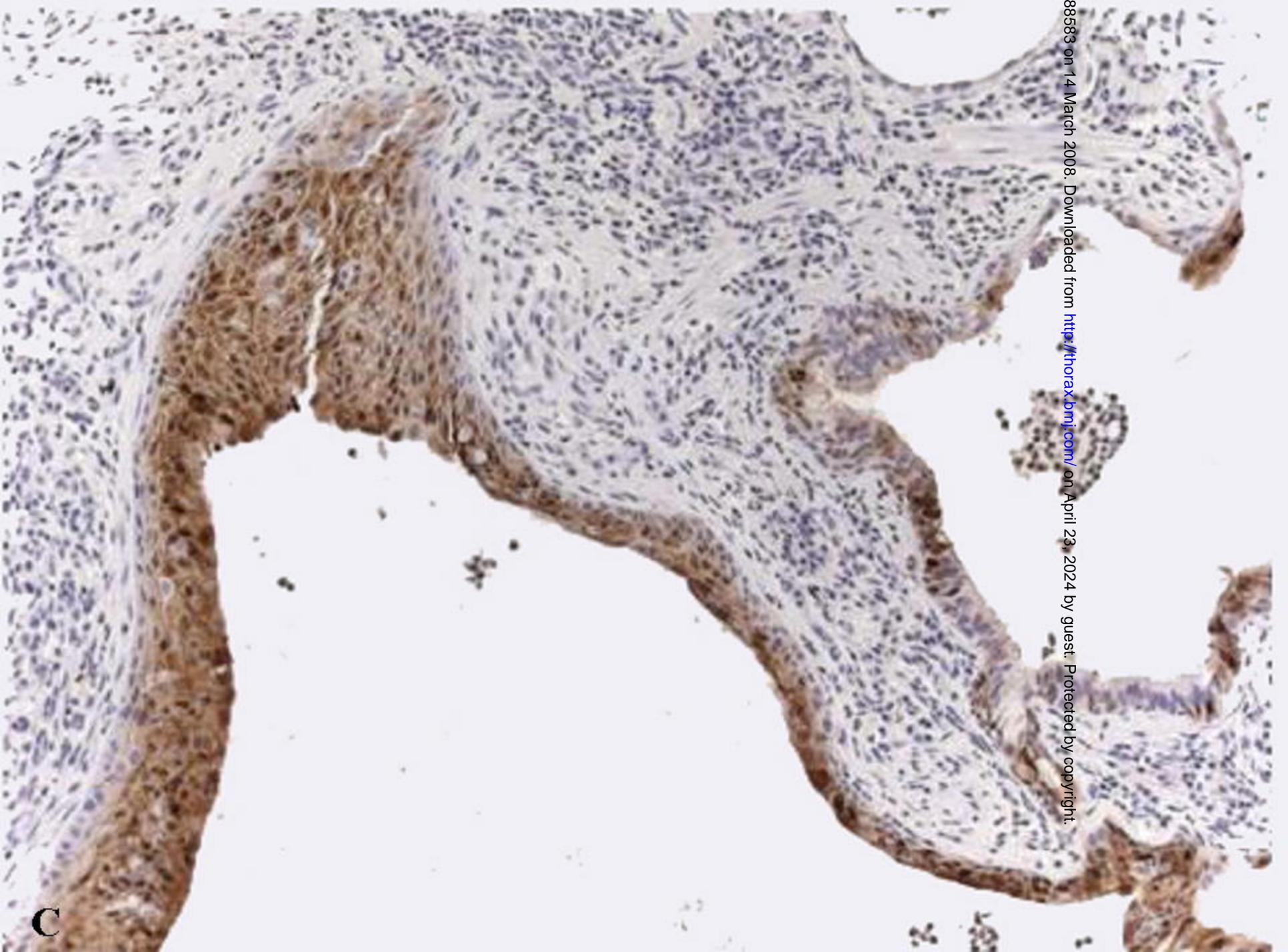
886863 on 14 March 2008. Downloaded from <http://thorax.bmj.com/> on April 23, 2024 by guest. Protected by copyright.

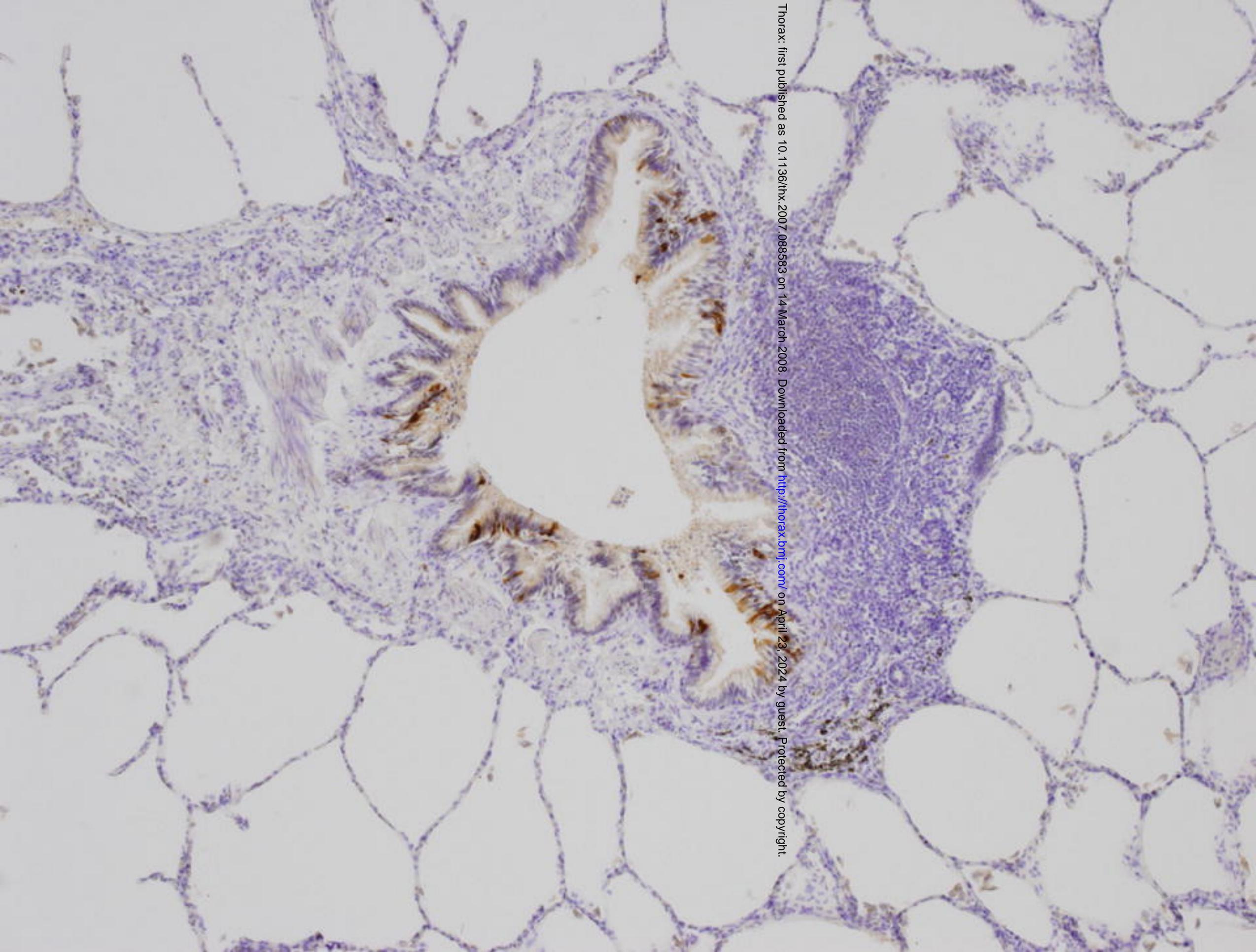
B

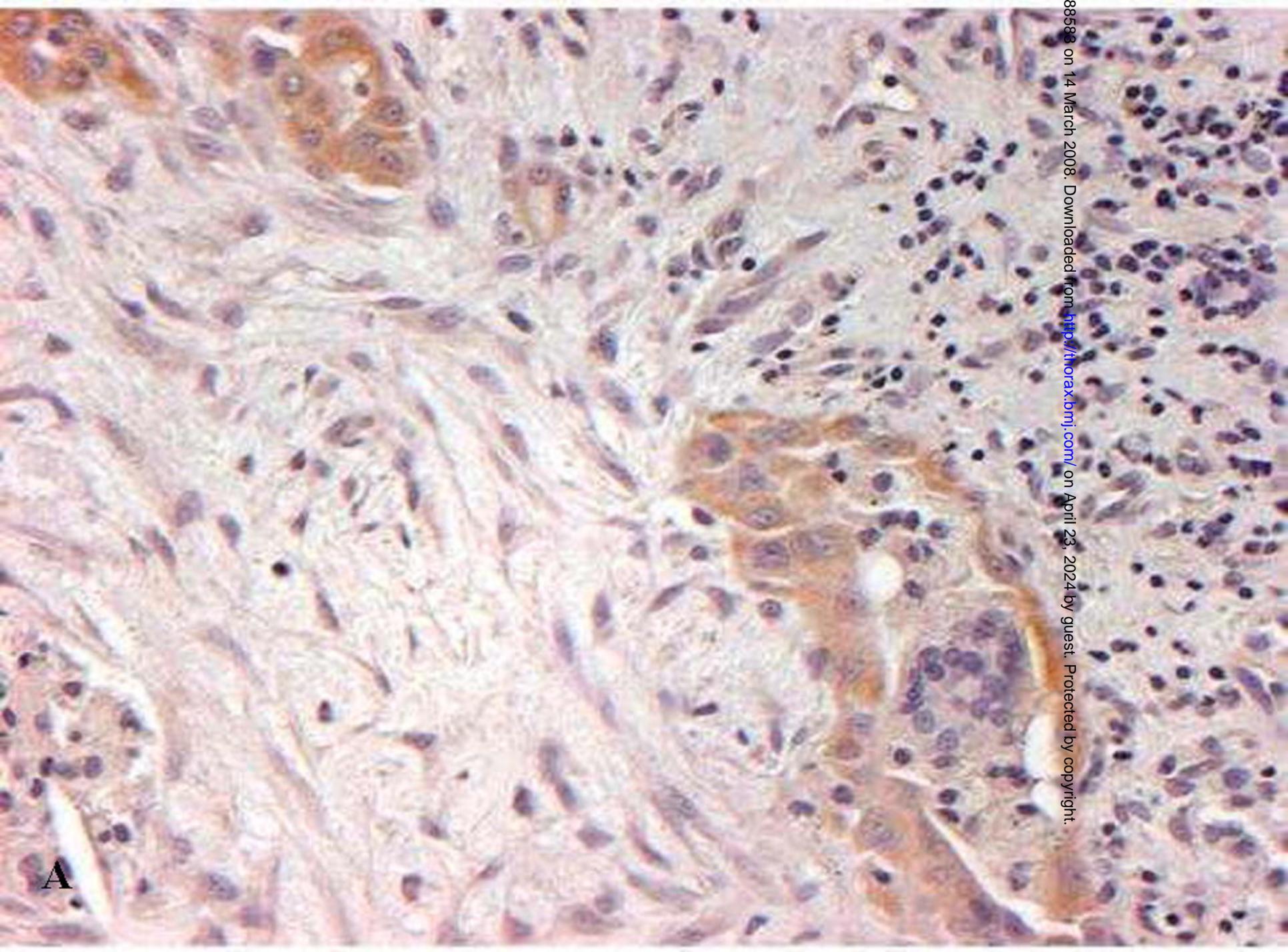




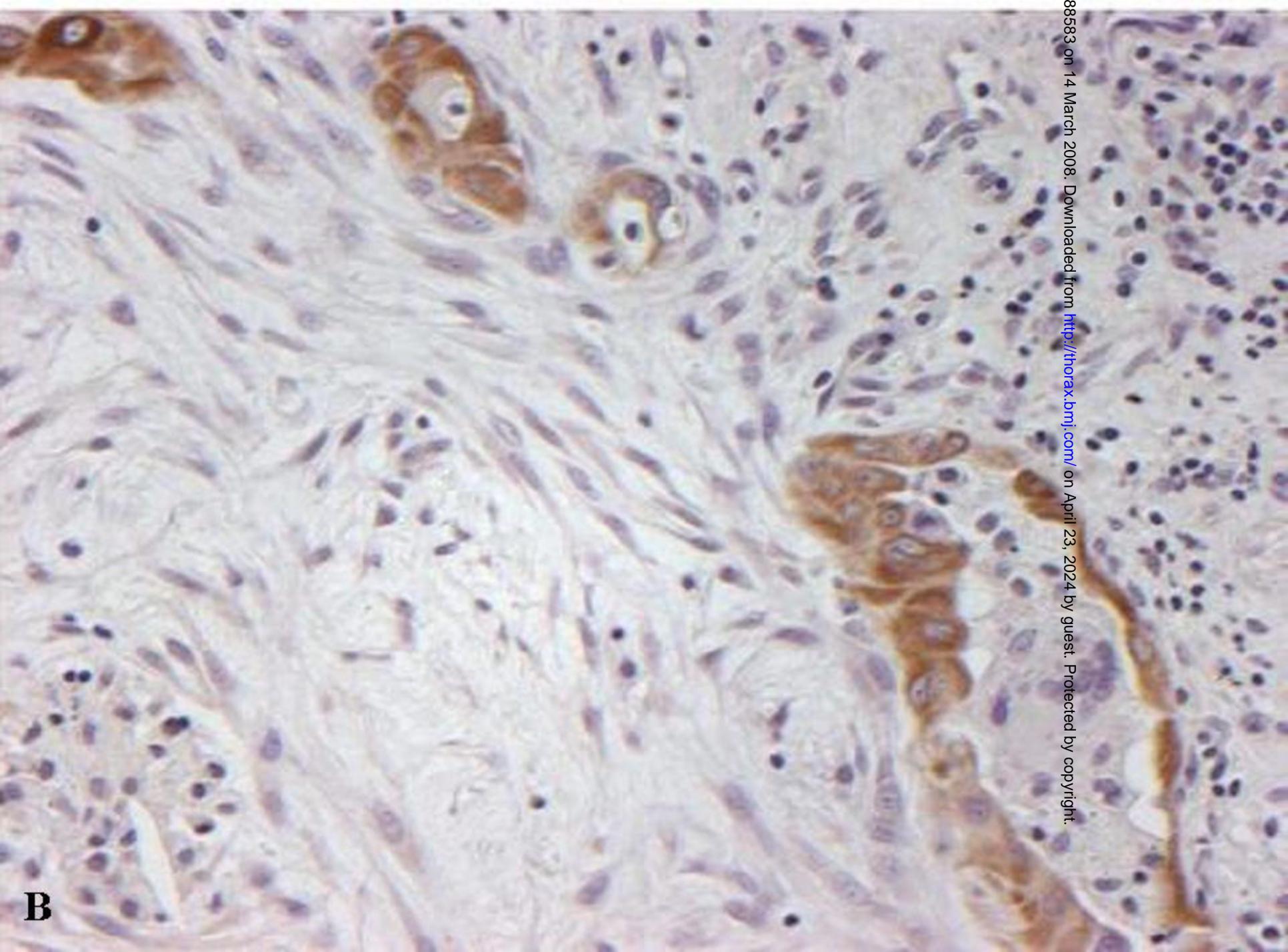
B





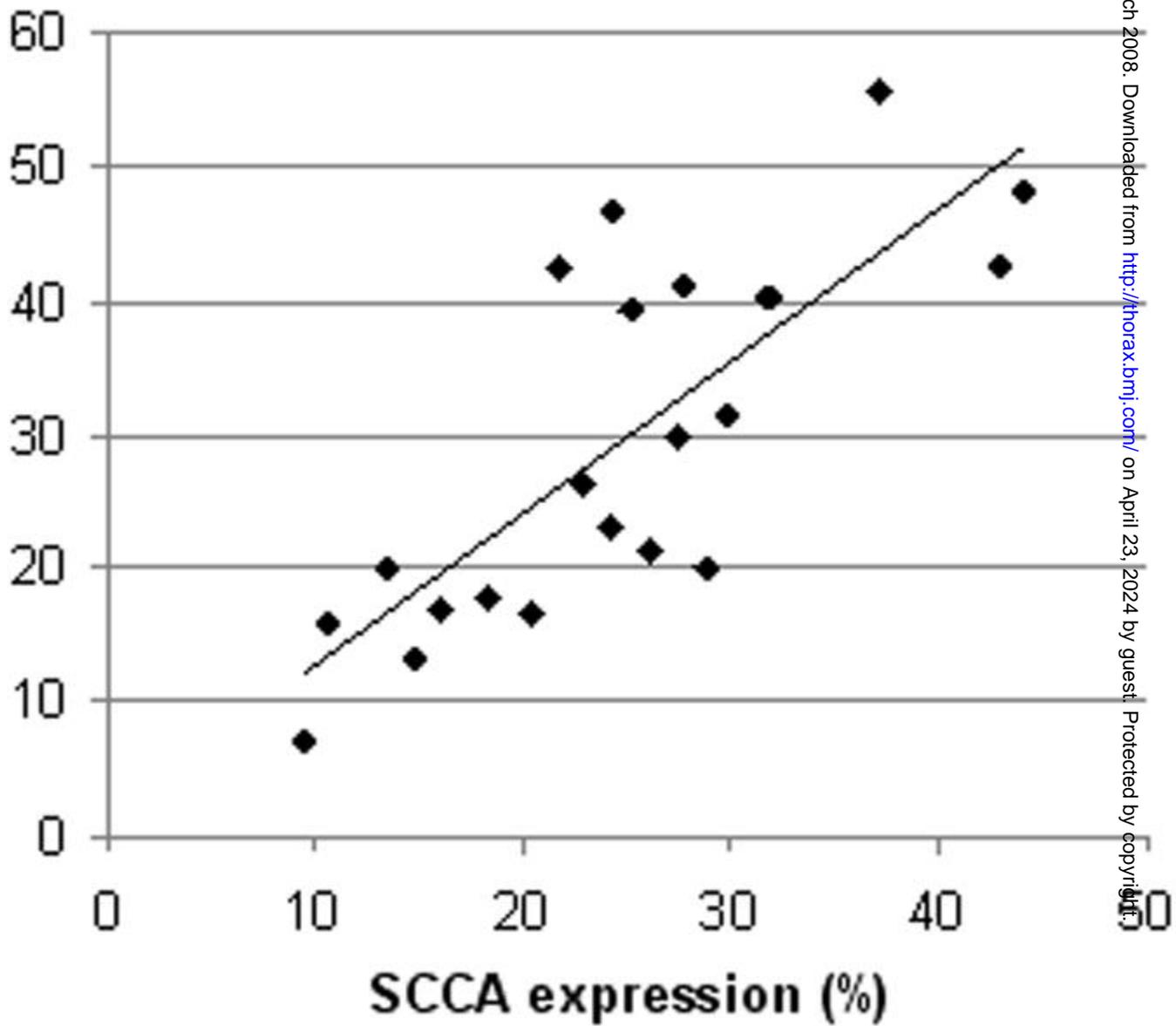


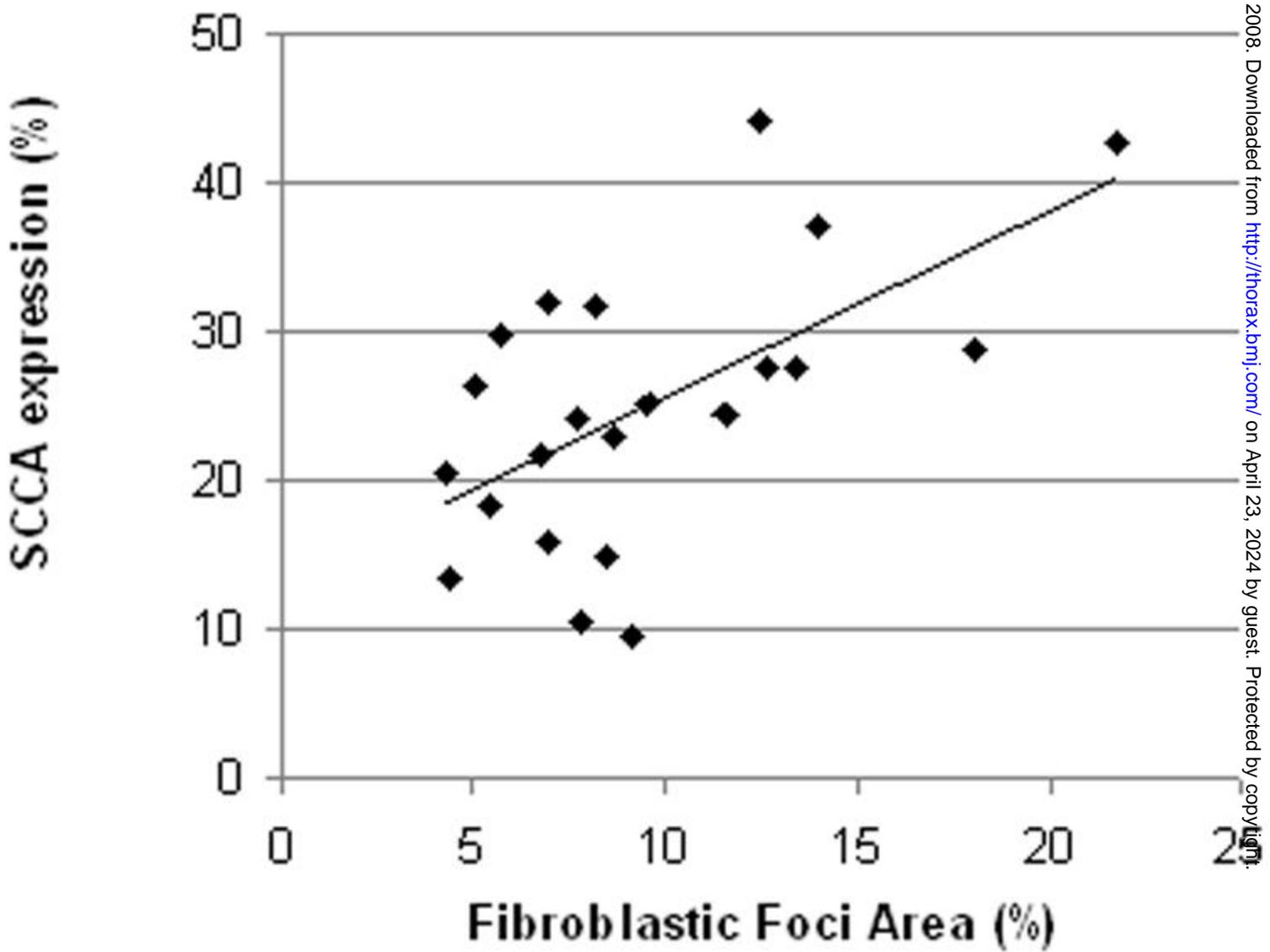
A



B

TGF β expression (%)





Δ DLCO (%)

