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# Impact of smoking on the expression of claudins in lung carcinoma

*Heta Merikallio* [a,*], *Riitta Kaarteenaho* [a,b], *Paavo Pääkkö* [c], *Siri Lehtonen* [a,d], *Pasi Hirvikoski* [c], *Riitta Mäkitaro* [b], *Terttu Harju* [a,b], *Ylermi Soini* [e]

[a] *Department of Internal Medicine, Respiratory Research Unit, Clinical Research Center, Oulu University Hospital, Oulu, Finland*
[b] *Institute of Clinical Medicine, Department of Internal Medicine, Respiratory Unit, Centre of Excellence in Research, University of Oulu, Oulu, Finland*
[c] *Department of Pathology, Oulu University Hospital, Oulu, Finland*
[d] *Department of Surgery, Oulu University Hospital, University of Oulu and Clinical Research Center, Oulu, Finland*
[e] *Institute of Clinical Medicine, Department of Clinical Pathology and Forensic Medicine, University of Eastern Finland, Kuopio, Finland*

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ABSTRACT

*Rationale:* Tight junctions regulate the paracellular permeability and orientation of cells and claudins are key components of tight junctions.
*Objectives:* To study the influence of cigarette smoke on claudin expression in vitro and in lung cancer patients.
*Methods:* We studied the effect of smoking on claudin expression by exposing a bronchial cell line (BEAS-2B) and two carcinoma cell lines (SK-LU1 and SK-MES1) to tobacco smoke for 48 h and analysed their claudin mRNA expression. The relation between smoked pack years and protein expression of claudins 1–5 and 7 in 344 lung cancer patients was determined by immunohistochemistry.
*Measurements and main results:* In BEAS-2B cells and SK-LU1 cells, an initial increase was followed by a decline in the mRNA expression of several claudins. In SK-MES1 cells, no evident elevation in claudin expression was observed.
Intense claudin 1 and 4 positivity was found more often in cancer samples of smokers and ex-smokers compared to non-smokers ($p < 0.001$ and $p = 0.003$, respectively). Heavy smokers with longer than 40 pack-years consumption had more often intense claudin 1 ($p = 0.011$), 4 ($p = 0.050$) or 7 ($p = 0.058$) expression in squamous cell carcinoma compared to non-smokers or smokers with fewer pack-years. Claudin 1 positivity predicted a better survival in adenocarcinoma ($p = 0.044$) and in squamous cell carcinoma ($p = 0.027$) and claudin 4 positivity in adenocarcinoma only ($p = 0.048$). In squamous cell carcinoma, claudin 7 positivity was associated with a better survival ($p = 0.011$).
*Conclusions:* Bronchial BEAS-2B cells and SK-LU1 cells respond to tobacco smoke by changing their claudin mRNA synthesis and resulting tight junction permeability changes may thus contribute to tobacco induced carcinogenesis both during initiation and progression. This concept is strengthened by findings in the clinical tumour material, where tobacco consumption was associated with claudin expression.



* *Corresponding author:* Address: Department of Internal Medicine, Respiratory Research Unit, Clinical Research Center, Oulu University Hospital, Aapistie 5A, FI-90220 Oulu, Finland. Tel.: +358 85376309; fax: +358 85376318.
E-mail address: hmerikal@paju.oulu.fi (H. Merikallio).
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## 1. Introduction

Tight junctions (TJs) are the most apical junctions in epithelial cells, forming a boundary between epithelial cell surface domains. They are involved in paracellular permeability and cell polarity.[1] Claudins are transmembrane proteins which are responsible for the backbone of the TJs. Claudins have four membrane-spanning parts, two extracellular loops and one intracellular loop, and N- and C-terminal cytoplasmic domains and they can form homo- or heterodimers to produce paired strands between cells, a property which determines the typical characteristics of different epithelial tissues. In general, claudins 2, 7, 10, 15 and 16 increase paracellular cation permeability by forming pores in the TJs whereas claudins 4, 5, 8, 11, 14 and 18 have a sealing function.[2] However, in airway TJs, claudins 1 and 3 decrease solute permeability whereas the opposite is the case for claudin 5.[3]

There are 24 members of the claudin family currently and their molecular weight is in the range of 20–27 kDa.[4] TJs and claudins are present in epithelial, endothelial and mesothelial cells, with tissue-specific distribution patterns.[5] In human carcinomas, a dysfunction of the TJ and dysregulation of claudins leads to a loss of cell–cell adhesion and disturbance in cellular differentiation, uncontrolled cell proliferation, a loss of cohesion and increased invasiveness.[4] Changes in the phenotype of TJs lead to increased influx of nutrients and growth factors, and provide an advantageous environment for tumour spread.[6] The expression of claudins varies in tumours at different sites[7] and is different in head and neck,[8] pancreatic,[9] colonic,[10,11] ovarian[12] and breast cancers[13] as well as within the same site like in gastric cancer, where there is lower expression of claudins in diffuse carcinoma compared to the intestinal type.[14] In some cases variation of claudin expression can be used in the differential diagnosis of tumours for instance claudins 3 and 4 expression between mesotheliomas and metastatic adenocarcinomas to the pleura.[15]

Normal human lung tissue expresses claudins 1–4 and 7 with differences in localizations and expression patterns i.e. claudin 1 is found in bronchial epithelial cells whereas claudin 3, 4 and 7 are positive also in type II pneumocytes of the alveolar epithelium.[16] In primary lung tumours, claudin 3 has been reported to be expressed less frequently in squamous cell carcinoma than in adenocarcinoma[17,18] and claudin 5 is reported to be more often expressed in adenocarcinomas than in squamous cell carcinomas.[18] According to Moldway and colleagues, the expression of claudin 7 was increased in all lung cancer subtypes except in neuroendocrine lung cancers.[17] Paschoud and colleagues detected a decreased expression of claudin 7 in adenocarcinomas and squamous cell carcinomas.[18]

Smoking is the most important cause of lung cancer. We hypothesised that tobacco smoke might influence claudin expression and alter TJ permeability and that these changes could contribute to tobacco-induced carcinogenesis. This hypothesis was tested by analysing the mRNA expression of claudins 1–5 and 7 in five different lung carcinoma cell lines (A427, A549, CALU-6, SK-MES -1 and SK-LU-1), in a human non-malignant bronchial cell line (BEAS-2B) and in two fresh tissue samples derived from a lung squamous cell carcinoma and adenocarcinoma. Furthermore, we investigated the effect of tobacco smoke exposure on the expression of claudins in BEAS-2B cells and two lung carcinoma cell lines (SK-MES-1 and SK-LU-1). Finally, the expression of claudins 1–5 and 7 was determined in different histological lung carcinoma types in a set of 344 array samples. The results were correlated to the clinical and histological data such as histological type, tumour size, spread of tumour, the presence of metastases and patient survival.

## 2. Materials and methods

### 2.1. Cell lines and fresh material

Human non-malignant bronchial cells and lung carcinoma cell lines (Table 1) were obtained from American Type Culture Collection (Rockville, MD). Total RNA was isolated from the cultured cells and two fresh tumour specimens for qRT-PCR.

### 2.2. Tobacco exposure experiments

BEAS-2B, SK-MES-1 and SK-LU-1 cells were exposed to cigarette smoke extract (CSE) at different time points. CSE was made by bubbling the smoke of two cigarettes through 50 ml of RPMI-1640 medium (Sigma-Aldrich, Steinheim,

**Table 1 – Primers used for real-time PCR.**

| | Name | Sequence | $T_m$ (°C) |
|---|---|---|---|
| Claudin 1 | Antisense | 5′-CGGGTTGCTTGCAATGTGC-3′ | 56 |
| | Sense | 5′-CCGGCGACAACATCGTGAC-3′ | |
| Claudin 2 | Antisense | 5′-TGGCAGAAGACTGTGCATCTC-3′ | 56 |
| | Sense | 5′-CAGCATTGTGACAGCAGTTGG-3′ | |
| Claudin 3 | Antisense | 5′-CCTTAGACGTAGTCCTTGCGG-3′ | 56 |
| | Sense | 5′-CGCGAGAAGAAGTACACGG-3′ | |
| Claudin 4 | Antisense | 5′-CAGCGCGATGCCCATTA-3′ | 56 |
| | Sense | 5′-CGCATCAGGACTGGCTTTATCTC-3′ | |
| Claudin 7 | Antisense | 5′-CAGGATGACTAGGGCAGACC-3′ | 58 |
| | Sense | 5′-AGGCATAATTTTCATCGTGG-3′ | |
| GAPDH | Antisense | 5′-GACAAGCTTCCCGTTCTCAG-3′ | 56 |
| | Sense | 5′-CCGGCGACAACATCGTGAC-3′ | |

Germany). Cells were exposed to 15% CSE for 2, 6, 24 and 48 h and control cells to RPMI-1640 medium. Cells were collected and total RNA was isolated for the RT-PCR.

### 2.3. Quantitative RT-PCR

The mRNA expression of claudins 1–4 and 7 was analysed by qRT-PCR from a normal bronchial cell line, five different lung carcinoma cell lines as well as two lung carcinoma samples. Cigarette smoke-exposed samples were also studied by qRT-PCR. The expressions of claudins were normalised with the housekeeping gene GAPDH and exposed cells were compared to control cells in each cell line. Results were calculated by the Likvak-method.[19] BEAS-2B cells were considered as the normal *in vivo* situation.

### 2.4. Tissue specimen

Three hundred and forty-four samples of lung carcinoma were retrieved from the archives of the Department of Pathology, Oulu University Hospital from years 1991 to 1996. (Table 2.) Table 3 shows TNM-data and the survival status of the patients with the two main lung carcinomas. All cases were re-evaluated by a pulmonary pathologist. From each sample two representative tumour regions were chosen and integrated into multi-tissue array blocks with Beecher Instruments Manual Tissue Arrayer (Beecher Instruments, Silver Spring, MD, USA). The presence of metastases and stage of the tumour were determined at the time of the operation. The survival data were obtained from the Finnish Cancer Registry.

### 2.5. Immunohistochemistry

The immunostaining for claudins was performed as previously described.[16,20,21] More detailed description is provided in the data supplement. In the evaluation, membrane bound positivity was considered significant. The immunostaining was assessed as 0 (no immunostaining) to 4 (76–100% positive cells).

**Table 3 – TNM-data and survival status in squamous cell carcinomas and adenocarcinomas.**

| | Squamous cell carcinoma | Adenocarcinoma |
|---|---|---|
| N | 128 | 108 |
| pT 1/2/3/4 | 19/72/19/0 | 28/55/14/4 |
| pN 0/1/2 | 58/39/15 | 54/25/20 |
| M 0/1 | 109/3 | 92/8 |
| Cause of death: lung carcinoma | 73 | 75 |
| Cause of death: other reason | 44 | 28 |
| Alive | 11 | 5 |

The evaluation was performed independently by two pathologists (RK, YS) who were blinded to knowledge of clinical data. In the evaluation of survival and other factors, the cases were further divided into two groups with weak or no positivity (0, <1) versus strong positivity ($\geqslant 1$).

### 2.6. Statistical methods

The statistical analyses were performed with SPSS for Windows software (SPSS, Chicago, IL, USA). Continuous data were compared using analysis of variance (ANOVA) followed by two-tailed *t*-tests. Categorical data were compared using Fisher's exact. Survival-data were analysed using the Kaplan–Meier method with the use of the log-rank test and only deaths with lung tumour as the primary cause of death were considered. *p*-Values less than 0.05 were considered statistically significant.

### 2.7. Ethical considerations

The study was approved by the ethical committee of Northern Ostrobothnia Hospital District. The study protocol was accepted by the Finnish Natiolegal board. The survival data

**Table 2 – Characteristics of cases studied in the different histologic subgroups.**

| | Squamous cell carcinoma | Adenosquamous carcinoma | Adeno-carcinoma | Bronchiolo-alveolar carcinoma | Small cell carcinoma | Large cell carcinoma | Carcinoid tumour | Metastases |
|---|---|---|---|---|---|---|---|---|
| N | 128 | 14 | 108 | 10 | 11 | 12 | 6 | 54 |
| Age years (SD) | 65 (8) | 69 (11) | 63 (9) | 62 (11) | 66 (8) | 63 (11) | 47 (10) | 54 (19) |
| Sex M/F | 119/9 | 12/2 | 77/31 | 6/4 | 9/2 | 10/2 | 4/2 | 27/27 |
| Pack-years(SD) | 43 (18) | 44 (12) | 32 (21) | 29 (36) | 36 (11) | 46 (26) | 11 (10) | 9 (17) |
| FEV1 l/s(SD) | 2.5 (0.65) | 2.16 (0.59) | 2.44 (0.76) | 3.13 (0.94) | 2.27 (0.57) | 2.57 (0.93) | 3.43 (0.73) | 2.74 (1.18) |
| FEV1% predicted(SD) | 74 (17) | 65 (12) | 76 (19) | 85 (34) | 70 (13) | 77 (19) | 97 (23) | 83 (25) |
| FVC1(SD) | 3.64 (0.82) | 3.42 (1.3) | 3.44 (1.3) | 4.28 (1.53) | 3.33 (0.83) | 3.77 (1.34) | 4.14 (0.77) | 3.72 (1.46) |
| FVC% predicted(SD) | 87 (17) | 83 (20) | 88 (16) | 102 (32) | 84 (12) | 93 (25) | 95 (19) | 93 (27) |
| FEV1/FVC(SD) | 71 (11) | 64 (11) | 73 (12) | 74 (10) | 68 (13) | 67 (12) | 85 (6) | 74 (9) |
| DCO(SD) | 6.4 (1.67) | 5.54 (0.72) | 6.73 (2.1) | ND | 6.0 (1.24) | 6.85 (2.26) | 8.59 (1.54) | 9.13 (1.64) |
| DCO% predicted(SD) | 79 (20) | 76 (8) | 83 (24) | ND | 71 (9) | 85 (21) | 100 (8) | 101 (14) |

*Abbreviations:* M, male, F, female, FEV, forced expiratory volume; FVC, forced expiratory vital volume; SD, standard deviation; DCO, diffusion capacity; ND, means not detected.

were obtained from the Finnish Cancer Register after receiving permission from the Ministry of Health and Social Welfare.

## 3. Results

### 3.1. Claudin mRNA expression

In all studied cell lines as well as in lung cancer samples, mRNA for studied claudins was detected by RT-PCR, with variable regulation. The results are compiled in Table 4. RT-PCR results were confirmed by gel electrophoresis. The expression of claudin 1 was very intense in all cell lines. In addition, fresh samples displayed very intense RNA expression. Relative to BEAS-2B cell lines, only A427 showed stronger mRNA expression. Claudin 2 was visualised only in A549 cells and SK-MES-1 cells. The expression of this claudin was very weak in the BEAS-2B cell line, and similarly the RNA level could not been detected visually at all on the gel. Equally strong claudin 3 mRNA expression was found in A427 and in SK-MES-1 cell lines. No apparent differences between these cell lines could on visual inspection be detected. Claudins 4 and 7 had also comparably intense expression levels in cell lines. SK-MES-1 expressed claudin 4 mRNA more intensely and the A427 line exhibited stronger expression of claudin 7 mRNA than BEAS-2B cells. Despite several attempts, the RT-PCR of claudin 5 was never successful. In Table 4 the relative differences of the samples are shown.

### 3.2. Cigarette smoke exposure for cell lines

BEAS-2B, SK-LU-1 and SK-MES-1 cell lines were chosen to be exposed to the cigarette smoke. The results of the experiments are compiled in Table 5. After 2 h of exposure the level of claudins 2–4 and 7 had increased in BEAS-2B cells. A major elevation of all studied claudins was seen in SK-LU1 cells whereas SK-MES1 cells did not display any evident changes. After 6 h exposure, there was a lowering in the expression of all claudins in BEAS-2B and SK-LU1 cells, except for claudin 2 which declined more slowly in the latter cell line. Again, SK-MES1 cells did not reveal any evident changes.

At the 24 h time point expressions of claudins 2–4 and 7 increased again in BEAS-2B cells but declined at the 48 h time point except for claudin 4 which continued to increase. In SK-LU1 cells, there was no such "secondary response" except for claudin 4 which, however declined, as did the other claudins at the 48 h time point. In SK-MES1 cells claudins 1 and 3 decreased slightly at the 24 and 48 h time points but there was a slight increase in claudins 4 and 7 expression at the 48 h time point.

**Table 4 – qRT-PCR results for cell lines (fold increase compared to corresponding claudin expression in BEAS-2B cells (SD)).**

| Sample | Claudin 1 | Claudin 2 | Claudin 3 | Claudin 4 | Claudin 7 |
|---|---|---|---|---|---|
| BEAS-2B | 1 (1.38) | 1 (3.13) | 1 (1.17) | 1 (0.80) | 1 (4.11) |
| A427 | 19 (30.3) | 2.6 (7.2) | 18.03 (19.73) | 0.07 (0.07) | 5.3 (2.38) |
| A549 | 0.4 (0.5) | 3625 (9938) | 3 (3.07) | 0.09 (0.07) | 0.02 (0.01) |
| CALU-6 | 1.1 (1.35) | 1.1 (3.65) | 2.2 (2.59) | 1.1 (0.83) | 1 (2.79) |
| SK-LU-1 | 0.004 (0.004) | 1 (3.34) | 0.09 (0.12) | 0.04 (0.03) | 0.01 (0.01) |
| SK-MES-1 | 1 (1.29) | 142 (474) | 16.7 (18.03) | 17 (6.73) | 0.11 (0.07) |
| Adenocarcinoma | 0.03 (0.03) | 0.7 (2.02) | 10.4 (12.86) | 0.6 (0.53) | 3 (1.77) |
| Squamous cell carcinoma | 0.07 (0.07) | 1.05 (2.91) | 59.4 (59.28) | 1.7 (1.47) | 5.1(3.33) |

**Table 5 – Results of cigarette smoke exposure. The results were calculated by the Likvak-method. In the Likvak-method, exposed cells were compared to control cells. The table reveals the relationship between control cells and exposed cells. Exposed cells express claudins more than control cells when the result is >1 and less when <1.**

| Cell line | Time (h) | Claudin 1 mean (SD) | Claudin 2 mean (SD) | Claudin 3 mean (SD) | Claudin 4 mean (SD) | Claudin 7 mean (SD) |
|---|---|---|---|---|---|---|
| BEAS-2B | 2 | 1.28 (1.10) | 1.42 (0.71) | 1.39 (0.79) | 1.75 (0.62) | 2.20 (2.66) |
| | 6 | 1.28 (1.00) | 0.57 (0.57) | 0.78 (0.59) | 1.68 (1.93) | 0.88 (0.98) |
| | 24 | 0.53 (0.22) | 1.83 (1.46) | 2.44 (0.81) | 2.83 (0.76) | 1.40 (0.59) |
| | 48 | 0.63 (0.29) | 0.65 (0.09) | 0.69 (0.49) | 3.90 (1.76) | 1.10 (0.43) |
| SK-LU1 | 2 | 44.43 (52.90) | 2.97 (2.27) | 28.88 (33.30) | 33.34 (39.39) | 1.97 (1.86) |
| | 6 | 2.91 (4.25) | 5.32 (8.03) | 4.07 (6.86) | 0.94 (0.37) | 5.81 (10.41) |
| | 24 | 1.10 (0.80) | 1.31 (1.04) | 0.73 (1.00) | 1.95 (1.19) | 1.29 (1.56) |
| | 48 | 0.26 (0.20) | 0.49 (0.42) | 0.32 (0.47) | 0.41 (0.35) | 0.39 (0.40) |
| SK-MES1 | 2 | 0.92 (0.40) | 0.98 (0.33) | 1.29 (0.39) | 0.86 (0.31) | 1.13 (0.48) |
| | 6 | 1.36 (1.10) | 1.12 (0.53) | 1.38 (1.24) | 1.12 (0.61) | 0.44 (0.18) |
| | 24 | 0.81 (0.17) | 1.20 (0.70) | 1.14 (0.99) | 17.05 (31.97) | 1.77 (1.80) |
| | 48 | 0.25 (0.15) | 0.90 (0.40) | 0.57 (0.13) | 1.41 (0.68) | 1.45 (0.82) |

### 3.3. *Association of smoking with claudin expression in the histological material*

In the whole tumour material, intense claudins 1 and 4 positivity was found more often in carcinoma samples of smokers and ex-smokers compared to non-smokers ($p < 0.001$ and $p = 0.003$, respectively). An association with pack-years was found in squamous cell carcinoma, heavy smokers with more than 40 pack-years having more often intense claudin 1 ($p = 0.011$), 4 ($p = 0.050$) or 7 ($p = 0.058$, approaching significance) expression compared to non-smokers or smokers with less pack-years. Interestingly in adenocarcinoma, there was a trend for more intense claudin 2 expression with less pack-years and weak or negative expression for those with more pack-years ($p = 0.053$). The association of pack years with claudin expression for the squamous cell carcinoma material has been shown in Fig. 1. Impaired lung function was associated with positive claudin 1 expression ($p = 0.032$ for FEV1 and $p = 0.006$ for DCO/VA) and claudin 7 expression ($p = 0.032$ for FEV1).

### 3.4. *Immunohistological expression of claudins in lung tumours of different histology*

The results for the immunohistochemical staining of claudins 1–5 and 7 are compiled in Table 6. There was extensive variation in the expression of claudins between different histological types of tumours. The three primary lung epithelial tumours (squamous cell carcinoma, adenocarcinoma and small cell carcinoma) exhibited significantly stronger claudin 1 positivity than the other types of lung tumours and metastases. Squamous cell carcinomas and adenocarcinomas had significantly more cases with claudin 2 positivity than small cell carcinomas or carcinoid tumours. Squamous cell carcinomas had a significantly lower claudin 3 positivity compared to adenocarcinomas (Fig. 3A), small cell carcinomas,

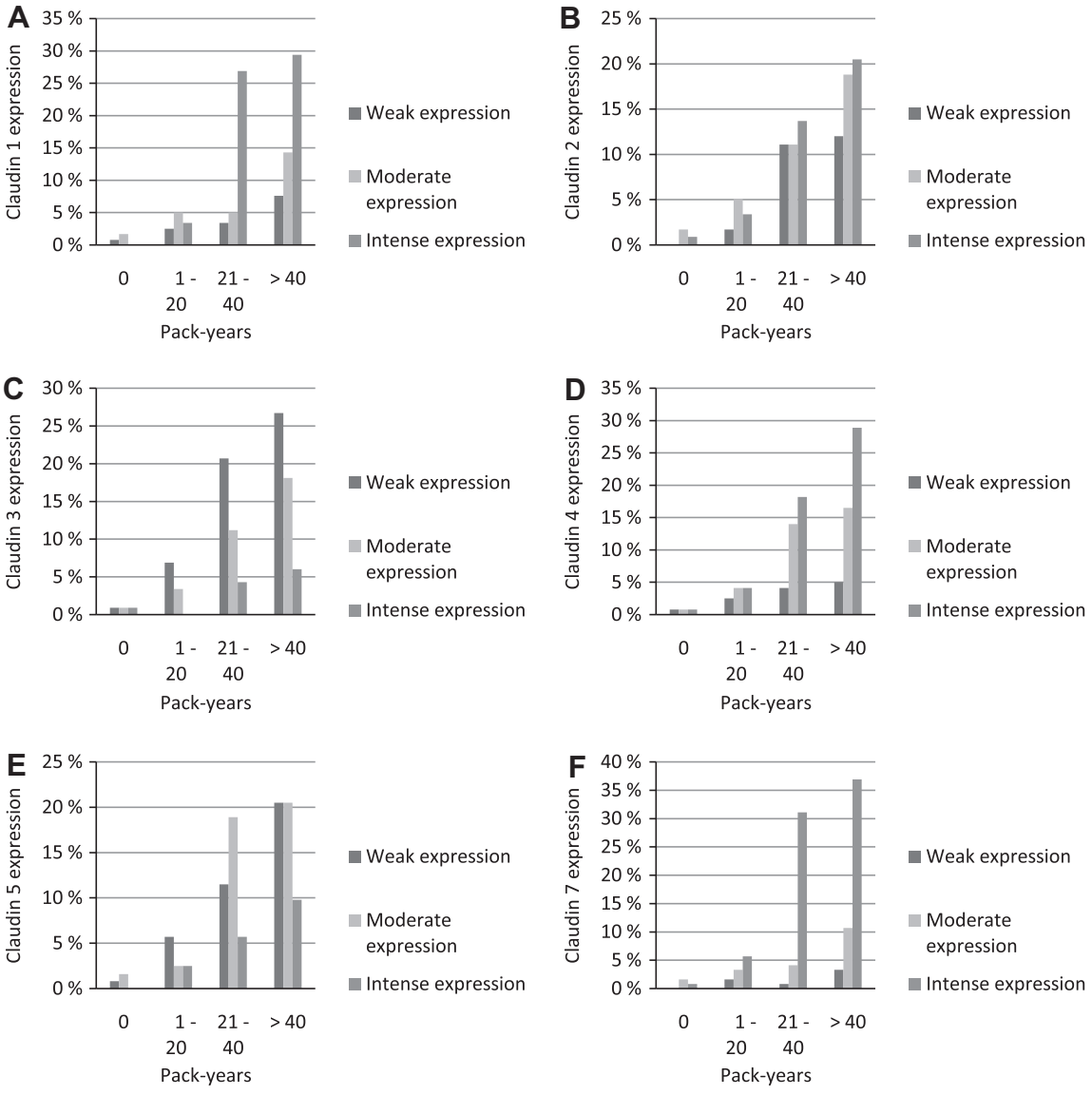


**Fig. 1 – Relationship between claudin expression and pack-years in squamous cell carcinoma specimen. (A) Claudin1 ($p = 0.011$), (B) claudin 2, (C) claudin 3, (D) claudin 4 ($p = 0.050$), (E) claudin 5 and (F) claudin 7 ($p = 0.058$).**

**Table 6 – Positive cases of immunohistochemical stainings.**

| Histology | Number of positive cases (%) | | | | | |
|---|---|---|---|---|---|---|
| | Claudin 1 | Claudin 2 | Claudin 3 | Claudin 4 | Claudin 5 | Claudin 7 |
| Squamous Cell Carcinoma | 109 (88) | 92 (75) | 54 (45) | 110 (88) | 77 (61) | 120 (94) |
| Adenosquamous Carcinoma | 8 (80) | 7 (70) | 7 (70) | 8 (80) | 4 (40) | 9 (90) |
| Adenocarcinoma | 93 (87) | 76 (76) | 98 (90) | 104 (94) | 84 (77) | 110 (99) |
| Bronchioloalveolar Carcinoma | 9 (100) | 8 (89) | 9 (100) | 8 (97) | 5 (56) | 9 (100) |
| Small Cell Carcinoma | 11 (92) | 3 (30) | 11 (92) | 11 (91) | 6 (50) | 11 (91) |
| Large Cell Carcinoma | 7 (58) | 6 (60) | 2 (25) | 9 (82) | 1 (10) | 7 (64) |
| Carcinoid tumour | 0 (0) | 3 (37) | 5 (56) | 3 (37) | 2 (25) | 5 (62) |
| Metastases | 24 (41) | 34 (69) | 26 (55) | 33 (63) | 20 (40) | 37 (71) |

bronchioloalveolar carcinomas, carcinoid tumours and adenosquamous carcinomas.

Small cell carcinomas exhibited stronger claudin 4 positivity compared to squamous cell carcinomas, large cell carcinomas, carcinoid tumours and other metastases In addition, adenocarcinomas (Fig. 3B) had more intense claudin 4 expression than squamous cell carcinomas (Fig. 2B), carcinoid tumours and other metastases. Compared to the other claudins studied herein, claudin 5 was clearly the most weakly expressed of all studied claudins (see Fig. 4). The strongest positivity, on the other hand, was for claudin 7 (see Table 6, Figs. 2D and 3D). All tumours, both primary and metastatic, displayed over 90% of intense positivity while large cell carcinomas and other lung tumours, containing mesenchymal tumours and metastatic lung tumours had a lower expression of claudin 7 squamous cell carcinoma. Other tumours and lung metastases also had less intense claudin 7 expression compared to squamous cell carcinomas, adenocarcinomas, small cell carcinomas and bronchioloalveolar carcinomas.

### 3.5. *Survival*

Claudin 1 positivity predicted better survival in adenocarcinoma ($p = 0.044$) and in squamous cell carcinoma ($p = 0.027$). (Fig. 5C and A). In cases of adenocarcinoma, positive claudin 4 staining predicted better survival ($p = 0.048$) but in squamous cell carcinoma no difference in survival was found (Fig. 5D). In squamous cell carcinoma, claudin 7 positivity was associated with better survival ($p = 0.011$) but not in adenocarcinoma (Fig. 5B).

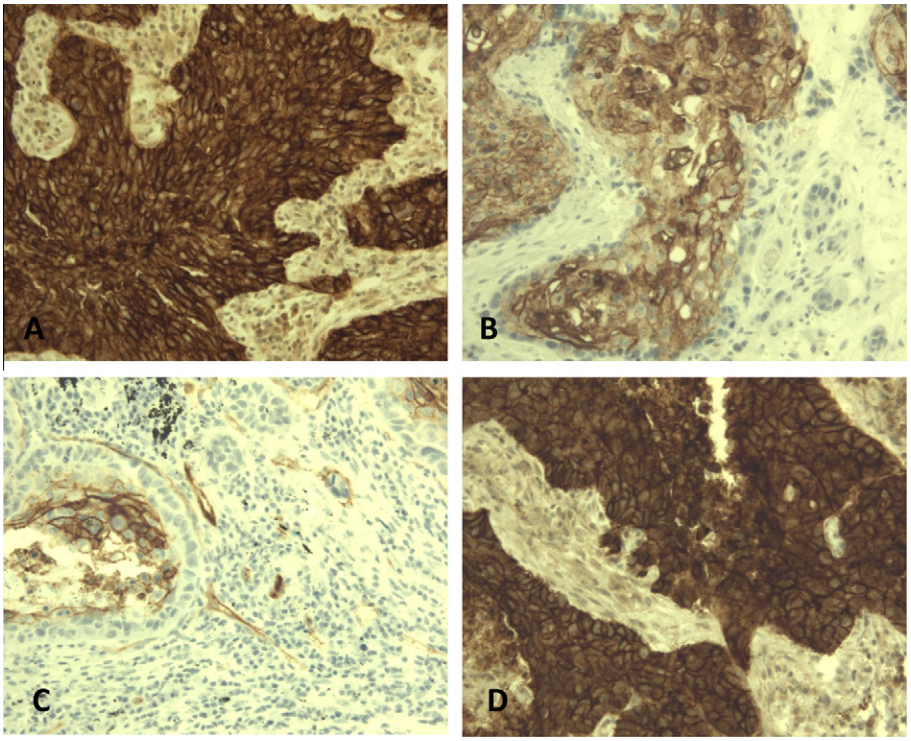


**Fig. 2 – Claudin expressions in squamous cell carcinomas. (A) Claudin 1 immunostaining: very strong expression, (B) claudin 4 immunostaining: strong expression, (C) claudin 5 staining: weak expression and (D) claudin 7 immunostaining: very strong expression.**

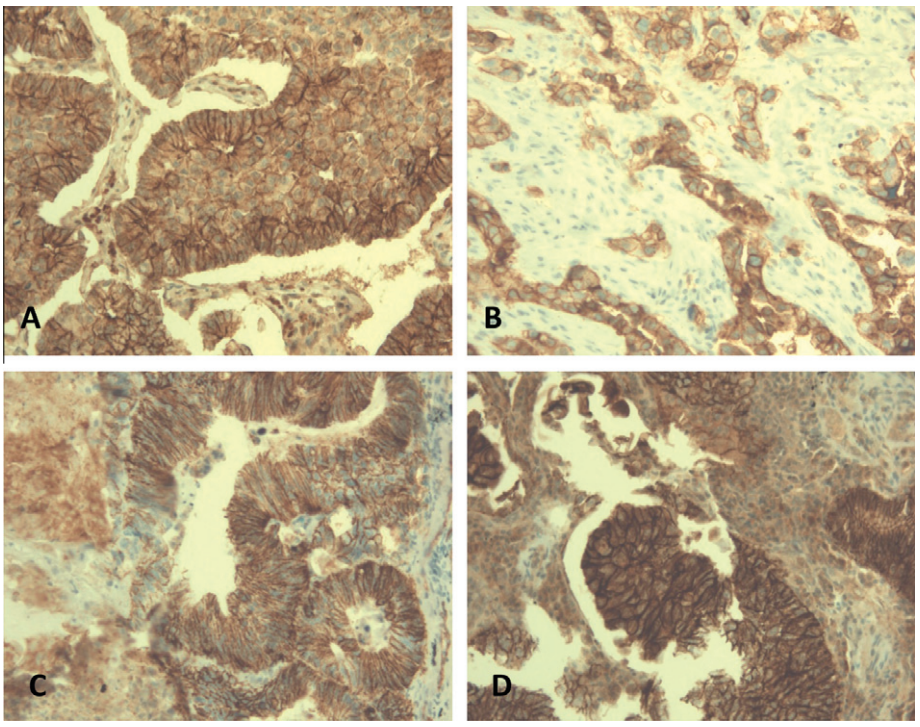


**Fig. 3 – Claudin expressions in adenocarcinomas. (A) claudin 3 immunostaining: very strong expression, (B) claudin 4 immunostaining: strong expression, (C) claudin 5 staining: strong expression and (D) claudin 7 immunostaining: very strong expression.**

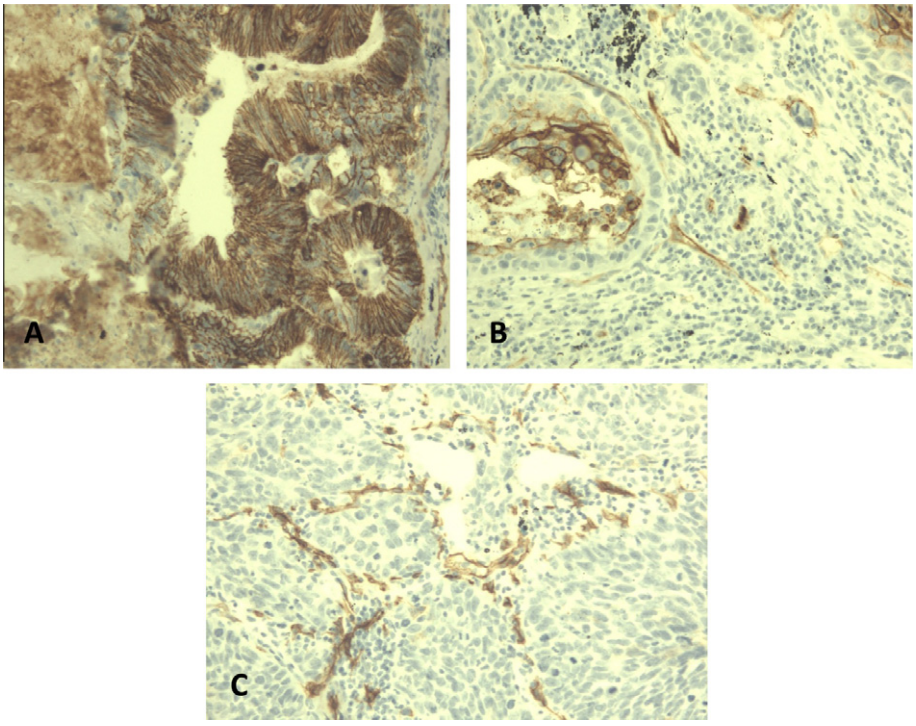


**Fig. 4 – Immunostaining of claudin 5 in different carcinomas. (A) strong claudin 5 expression in adenocarcinoma, (B) moderate claudin 5 expression in squamous cell carcinoma and (C) negative expression in small cell carcinoma, where only the blood vessels are seen to be expressing claudin 5.**

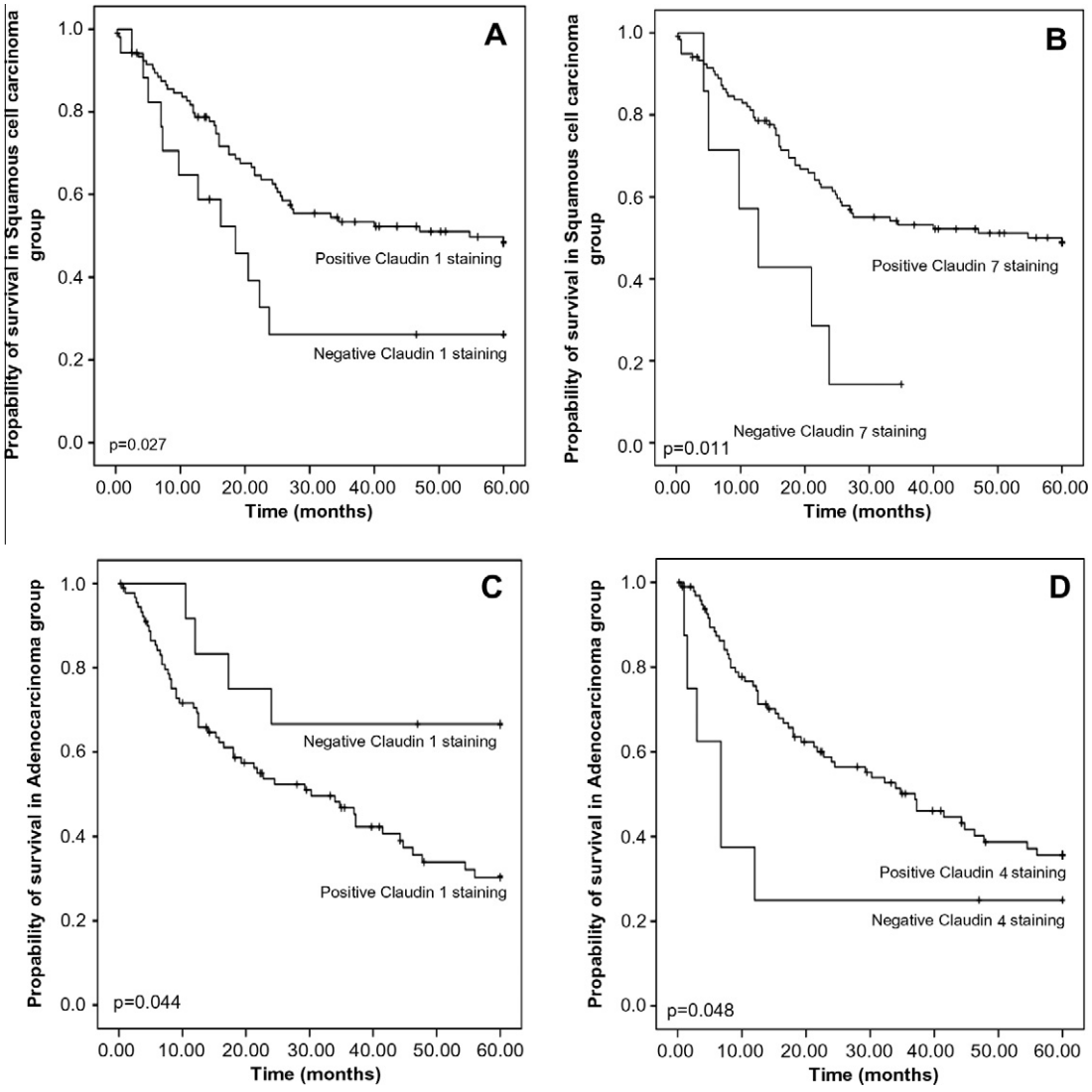


**Fig. 5 – Kaplan–Meier estimates of survival in squamous cell carcinomas and adenocarcinomas. (A) Survival association to claudin 1 expression in squamous cell carincoma. (B) Claudin 7 expression in squamous cell carcinoma group. Negative claudin 7 expression in squamous cell carcinomas was also associated with shorter survival as claudin 4 in adenocarcinomas. (C) Claudin 1 expression in adenocarcinoma group. (D) Claudin 4 expression in adenocarcinomas. Negative claudin 4 expression was associated with a shorter survival of the patients.**

### 3.6. *Other associations*

Carcinoma cases with negative or weak claudins 1, 4, 5 or 7 expression were younger compared to cases with strong claudin positivity ($p < 0.001$). For claudin 1, a small tumour size was associated with negative or weak expression ($p = 0.007$). There was a trend for strong claudin 7 expression in small T1 tumours and an association between strong expression and non-metastatic carcinoma ($p = 0.036$).

## 4. Discussion

We investigated the impact of smoking on the expression of claudins 1–5 and 7 non-neoplastic (BEAS-2B) and neoplastic cultured cells (SK-LU1, SK-MES1) exposed to tobacco smoke, and a large clinical material consisting of 344 lung tumour specimens where we studied the influence of tobacco consumption on claudin expression in lung tumours. Several cell lines were additionally studied for the expression of claudin mRNAs to detect putative variations in the expression of claudins between the cell lines.

Non-neoplastic BEAS-2B showed an increase in claudin expression after 2 h of exposure to tobacco smoke followed by a decline at 6 h (see Table 5). The synthesis of claudins was further increased at 24 h and further declined at 48 h. In SK-LU1 cells a clear peak in the levels could be seen at 2 h followed by a decline but in SK-MES1 cells no evident peaks were detected. When exposed to tobacco smoke BEAS-2B cells undergo a change in their TJ permeability which is reflected by the alterations observed in their claudin mRNA synthesis. Under *in vivo* conditions, this tight

junctional change would protect the adjacent bronchial tissue from toxic damage caused by tobacco smoke.[22]

When compared to the BEAS-2B cells a different pattern of claudin synthesis could be seen in the two neoplastic lung carcinoma cell lines. SK-LU1 cells displayed a strong response of claudin mRNA synthesis followed by a decline in claudin expression and SK-MES1 cells did not show any evident changes in the mRNA synthesis of the claudins studied. These results indicate that claudin expression, also in its response to tobacco smoke, is dysregulated in neoplastic cell lineages. The difference in the expression of claudins between these two carcinoma cell lines may be partly due to their different cell lineage origin. SK-LU1 cells harbour adenocarcinomatous features while SK-MES1 originates from squamous epithelial cells. It is known that the synthetic activity and distribution of claudins differ in glandular and squamous epithelia.[23] Adenocarcinoma cells are believed to resemble BEAS-2B bronchial epithelial cells, as was also the case in our tumour cell lines.

Tobacco smoke contains many chemicals but the most detrimental in terms of cancer development are the polycyclic aromatic amines (PAHs) in particular benzpyrene.[24] In a previous study, exposure of A549 cells to benzpyrene evoked an up-regulation of epithelial–mesenhymal transition (EMT) inducing genes such as twist[25] and exposure of colon carcinoma cells HT29 and LDL-1 to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) caused an up-regulation of snail and ZEB1.[26] In previous studies, it has been shown that transcriptional factors inducing EMT changes such as snail, slug, twist or ZEB1 may down-regulate the expression of adhesion molecules such as E-cadherin or claudins.[27] Carcinogens in tobacco smoke may thus directly influence the expression of crucial transcription factors and contribute to the carcinogenesis of lung tumours.

Further evidence for the significance of claudins in the pathogenesis of tobacco related lung cancer is seen in our results of the relationship between pack-years smoked by the patients and claudin expression in lung tumours. The expression of claudins 1 and 4 was more often found in lung tumours of smokers and ex-smokers and furthermore, expression of claudins 1, 4 and 7 were associated with more pack-years in squamous cell carcinoma. Conversely, a low claudin 2 expression was associated with more pack-years in adenocarcinoma.

In our study, different histologic types of lung carcinoma showed significant differences in claudin expression. This is in line with previous studies on lung carcinoma and with studies on carcinomas at other sites. This was observed in the expression of claudins in cell lines (see Table 4) where claudins exhibited a large individual variation of expression in different types of cell lines compared to BEAS-2B cells. According to the results, squamous cell carcinoma and adenocarcinoma strongly express different claudins and differ mainly in their expression of claudin 3 which has been determined also in other studies[17] and in tumours at other sites such as oesophageal carcinoma.[28] Squamous cell carcinoma and adenocarcinoma showed a higher expression of claudin 2 than the other types of lung carcinomas. Squamous cell carcinoma, adenocarcinoma and other types of primary lung tumours could be crudely separated by the use of claudins 2 and 3. Large cell carcinoma and squamous cell carcinoma could be separated by claudin 7 the expression of which was significantly lower in large cell carcinoma.

Epithelial pulmonary metastases displayed a slightly lower expression of claudins compared to the main types of primary pulmonary carcinomas, in particular, squamous cell carcinomas and adenocarcinomas. They had a significantly lower expression of claudins 1 and 7. The results are in line with previous studies showing alteration in adhesion molecules such as claudins 4 and 7 during the metastatic process.[29] In line with this hypothesis, our results revealed the tendency of metastatic cells to lose some of their adhesion features in the process of metastatic spread which is related to EMT and directed by transcriptional factors such as slug, snail or twist which have been shown to down-regulate certain claudins e.g. claudin 1.[30] Sarcomatoid tumours or sarcomas metastatic to the lung, not surprisingly, had an even lower expression of claudins with an expression level comparable to that found in carcinoid tumours. In previous studies, it has been shown that the expression of claudins is very low in mesenchymal tumours, a finding which also is observed in this study.[7] Claudin expression is evidently modified by the histological origin of the lung tumour in question thus reflecting the expression of the corresponding non-neoplastic cell. While EMT related transcription factors modulate the expression of claudins via promotion of EMT, the relations between these factors become more complex. Moreover, EMT-related markers like Zeb1 are also regulated by microRNAs[31] and little is known about their role in the regulation of EMT related markers or claudins in lung carcinoma.

In carcinomas of different origin, claudin expression has been found to show an association with prognosis in breast,[13] ovarian,[5,12] gastric[7] and renal clear cell carcinoma.[32] According to our results, claudins 1, 4 and 7 appeared to have prognostic significance in lung carcinoma. Strong claudins 1 and 7 expression predicted a better survival in squamous cell carcinoma whereas, it was claudins 1 and 4 which where diagnostic in adenocarcinoma. This is in line with a recent finding by Chao et al.[33] where there was low claudin 1 expression in those lung adenocarcinoma patients with shorter survival and *in vitro*, over-expression of claudin 1 inhibited cancer cell dissociation, migration and invasion. Chao and colleagues examined overall survival; we were fortunate to have access to data on the causes of death from the cancer register and thus in the survival analysis, only lung cancer deaths were recorded. On the other hand, low claudin 4 expression was associated with a positive pN-status and a shorter survival in patient with adenocarcinomas. This reflects the importance of claudin 4 in determining invasiveness, metastatic potential and survival also in lung adenocarcinoma, as has been recently noted in gastric cancer.[34] Low claudin 7 expression was associated with a large size of tumour in the whole material and shorter survival for squamous cell carcinoma. Claudin 7 could play a role in the regional growth of lung tumours whereas in squamous cell carcinoma there are also other factors determining prognosis.

In conclusion, we have shown that exposure to tobacco smoke may induce both short term and long term changes in TJ composition and function both in non-neoplastic and

neoplastic cell lines derived from lung. These kinds of changes may contribute to both initiation and progression of lung cancer. The claudins exhibit a wide variety of expression patterns in different types of lung tumours making them putative markers for tumour differentiation and in some cases also for patient prognosis.

## Conflict of interest statement

None declared.

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