Selection of potential targets for stratifying congenital pulmonary airway malformation patients with molecular imaging: is MUC1 the one?

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MUC1 is positive in mucinous proliferations in congenital pulmonary airway malformation patients and could stratify them into high- and low-risk groups with targeted molecular imaging, a noninvasive method to change the treatment strategy. https://bit.ly/462Z7fc

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Abstract

Currently there is a global lack of consensus about the best treatment for asymptomatic congenital pulmonary airway malformation (CPAM) patients. The somatic KRAS mutations commonly found in adult lung cancer combined with mucinous proliferations are sometimes found in CPAM. For this risk of developing malignancy, 70% of paediatric surgeons perform a resection for asymptomatic CPAM. In order to stratify these patients into high- and low-risk groups for developing malignancy, a minimally invasive diagnostic method is needed, for example targeted molecular imaging. A prerequisite for this technique is a cell membrane bound target. The aim of this study was to review the literature to identify potential targets for molecular imaging in CPAM patients and perform a first step to validate these findings.

A systematic search was conducted to identify possible targets in CPAM and adenocarcinoma *in situ* (AIS) patients. The most interesting targets were evaluated with immunofluorescent staining in adjacent lung tissue, $KRAS^+$ CPAM tissue and $KRAS^-$ CPAM tissue.

In 185 included studies, 143 possible targets were described, of which 20 targets were upregulated and membrane-bound. Six of them were also upregulated in lung AIS tissue (CEACAM5, E-cadherin, EGFR, ERBB2, ITGA2 and MUC1) and as such of possible interest. Validating studies showed that MUC1 is a potential interesting target.

This study provides an extensive overview of all known potential targets in CPAM that might identify those patients at risk for malignancy and conducted the first step towards validation, identifying MUC1 as the most promising target.

Introduction

Currently, a global lack of consensus exists about the best treatment for asymptomatic congenital pulmonary airway malformation (CPAM) patients. CPAM is increasingly detected due to improved prenatal imaging techniques such as the routine mid-trimester ultrasound scan, and sometimes even with the first-trimester scan [1, 2]. With a reported incidence of 1:7200 births, CPAM is the most common of all congenital lung anomalies [3]. CPAM patients can present with pulmonary symptoms such as pulmonary infections, cough or even need for oxygen support. Reported numbers of developing symptoms range from 3% to 64% (follow-up duration varied from 1 month to several years) [4–8]. Most CPAM patients (91–97%) are asymptomatic at birth, but can develop symptoms later in life. The treatment of these asymptomatic patients is either (prophylactic) surgery or watchful waiting.

Most surgeons (~70%) choose (prophylactic) resection as treatment for these patients [9–11]. The increasing rate of (recurrent) pulmonary infections [12] and the risk of malignant degeneration of CPAM tissue [13] are well known arguments for resection. CPAM can be divided into five histological subtypes [14], of which CPAM type 1, the most common variant, originates from the bronchi and consists of large cysts containing mucinous cells. 30% of all CPAM type 1 cases develop mucinous proliferations and the current hypothesis is that these mucinous proliferations can degenerate into adenocarcinoma *in situ* (AIS), formerly included in tumours defined as bronchioalveolar carcinoma [15–17].

In contrast, other specialists plead for monitoring asymptomatic CPAM patients instead of surgery because an operation is associated with potential complications such as infection, air leakage and bleeding and airway compression caused by resection, while long-term patient benefit is doubtful [18]. Currently, it is not possible to predict which patients are at risk of developing a malignancy, and treating all asymptomatic patients with surgery is a possible overtreatment in the majority of patients [19].

To establish a suitable and uniform treatment for the large group of asymptomatic CPAM patients, a stratification method is needed to divide these patients into “low”- and “high”-risk groups for the probability of developing malignancy later in life. Currently, with the existing diagnostic imaging modalities, this is impossible. Newly emerging molecular functional imaging techniques, such as targeted molecular imaging, could fill this gap [20, 21]. Targeted molecular imaging can be used in contrast-enhanced transabdominal ultrasound and endoscopic ultrasound, computed tomography (CT), magnetic resonance imaging, positron emission tomography (PET), photoacoustic imaging, fluorescence molecular imaging and Raman optical imaging [22]. A volumetric inspiratory chest CT scan is the current noninvasive golden standard for diagnosing CPAM in pre-operative patients, and targeted molecular PET-CT could potentially be a promising imaging technique for making a distinction within different CPAM patients groups. For this technique, a targeting ligand is needed directed against a specific target on the tissue of interest [23]. Preferably, such a target is upregulated in diseased tissue and bound to the plasma membrane. A schematic overview of this principle is shown in figure 1. In order to stratify CPAM

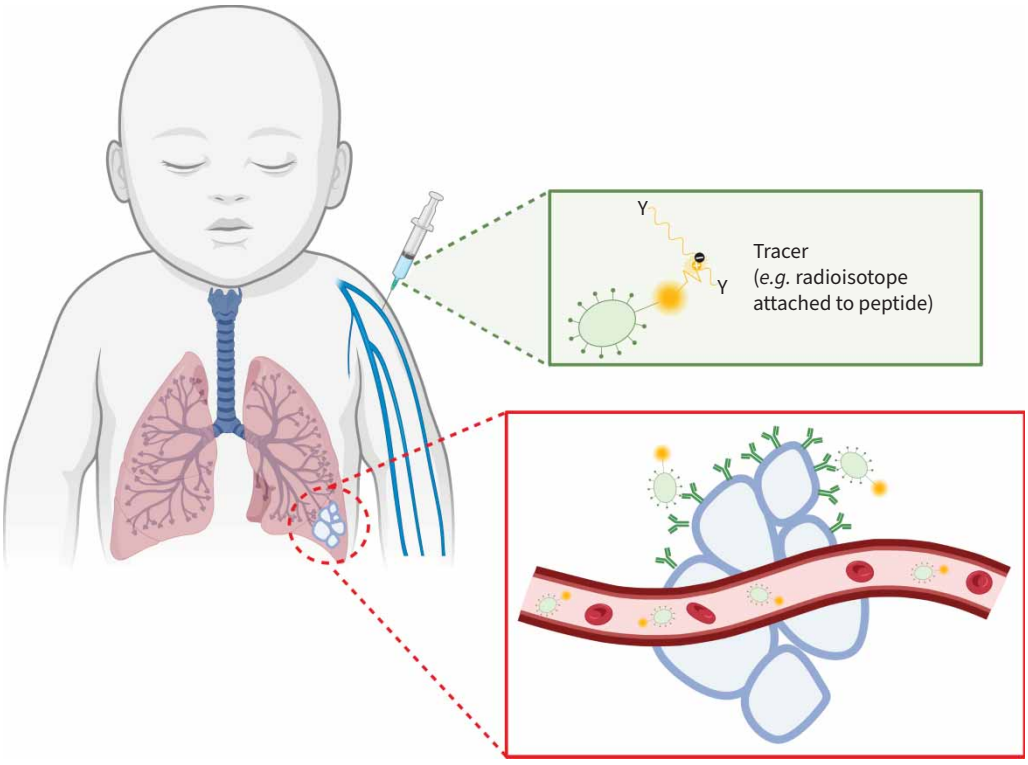

FIGURE 1 Schematic overview of the principle of tumour-targeted positron emission tomography imaging. A suitable tracer will be administered into the subject (*e.g.* a baby with a congenital pulmonary airway malformation). Depending on the size of the tracer, the tracer can target the cancer at multiple locations, *e.g.* intravascular, receptors on the cell membrane or intracellular. Figure created using BioRender.com.

patients using this novel technique, a membrane-bound target is needed that sufficiently discriminates between patients with high and low risk of developing malignancy.

Previously, we showed that mucinous proliferations in CPAM type 1 and type 2 patients is associated with KRAS mutations also known from adults with lung cancer. KRAS mutations were found in all mucinous proliferation tissue, whereas the surrounding healthy lung tissue did not contain this mutation [24–26]. Specifically, KRAS mutations located on exon 2 G12V and G12D were found in both CPAM and mucinous AIS [17, 25, 27–31]. Because KRAS mutations found in lung tissue in adults supports the diagnosis of malignancy, we hypothesised that KRAS-positive CPAM patients (*i.e.* the ones that show a KRAS mutation) belong to the high-risk group. KRAS mutations can cause the RAS protein to be constitutively activated. However, the RAS protein is not expressed on the outside of the plasma membrane and is therefore not a suitable candidate for targeted molecular imaging [32, 33]. As such, we aim to identify membrane-bound molecules associated with KRAS-mutant cells that could subsequently be used as targets in molecular imaging to stratify CPAM patients.

Therefore, the goal of this study was to analyse and describe the existing literature to identify potential targets for molecular imaging in (a)symptomatic CPAM patients and to verify these potential targets. Hence, the following research question was formulated: can we identify cell-surface targets that contribute to the stratification of CPAM patients into "high-risk" and "low-risk" groups for the development of malignancy later in life?

Materials and methods

A scoping review was conducted to explore the existing knowledge of targets associated to CPAM and pulmonary adenocarcinoma *in situ*, both in relation to KRAS mutations. The review was performed and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for scoping reviews guidelines [34]. An *a priori* designed study protocol was registered to Open Science Framework on 28 January 2022. The final protocol can be retrieved at osf.io/ku6jz.

Search

A systematic search strategy was developed in Embase by a biomedical information specialist from the medical library at our centre. This resulted in two search strings. The first query (S1) searched for targets in CPAM tissue. The second query (S2) searched for targets in adenocarcinoma of the lung (figure 2). The searches were performed in Embase, MEDLINE, Web of Science, Cochrane Central Register of Controlled Trials and Google Scholar on 30 November 2021.

Eligibility criteria

To be included, articles needed to fulfil the following criteria: 1) studies from S1 had to describe targets in tissue from CPAM patients (children or adults); 2) studies from S2 had to describe targets in tissue from children or adults with AIS of the lung (with or without KRAS mutation). Exclusion of the article followed whenever one of the following criteria was met: 1) only describing congenital lung anomalies other than CPAM (S1); 2) solely invasive forms of adenocarcinoma of the lung (S2); 3) only describing types of malignancy other than adenocarcinoma of the lung (S2); 4) no (relevant) targets mentioned in the article, for example blood/serum targets, physiological targets, radiographic targets and targets extracted from sputum, pleural effusion, tracheal aspirates, bronchoalveolar lavage or bronchial brushing, are not usable for molecular imaging and are therefore irrelevant in this scoping review (S1, S2); 5) full text unavailable (S1, S2); 6) non-English articles (S1, S2); 7) editorials/letter to the editors (S1, S2). No publication year restrictions were applied.

Study selection

Duplicate articles were removed. Each result was screened on title, abstract and keywords by two independent reviewers (C. van Horik, M.J.P. Zuidweg). After this screening was completed by both reviewers, the results were compared and discussed until consensus was reached. A third reviewer (W.S.F.J. Tummers) was consulted when consensus could not be reached. The full text of the references selected based on titles, abstracts and keywords were retrieved for further (full-text) selection based on the inclusion and exclusion criteria by two investigators (C. van Horik, M.J.P. Zuidweg). Disagreement on inclusion/exclusion based on full text of the articles was resolved by discussion. All articles recovered from S2 that only mentioned CPAM and no adenocarcinoma were excluded, provided that these articles were also found in S1.

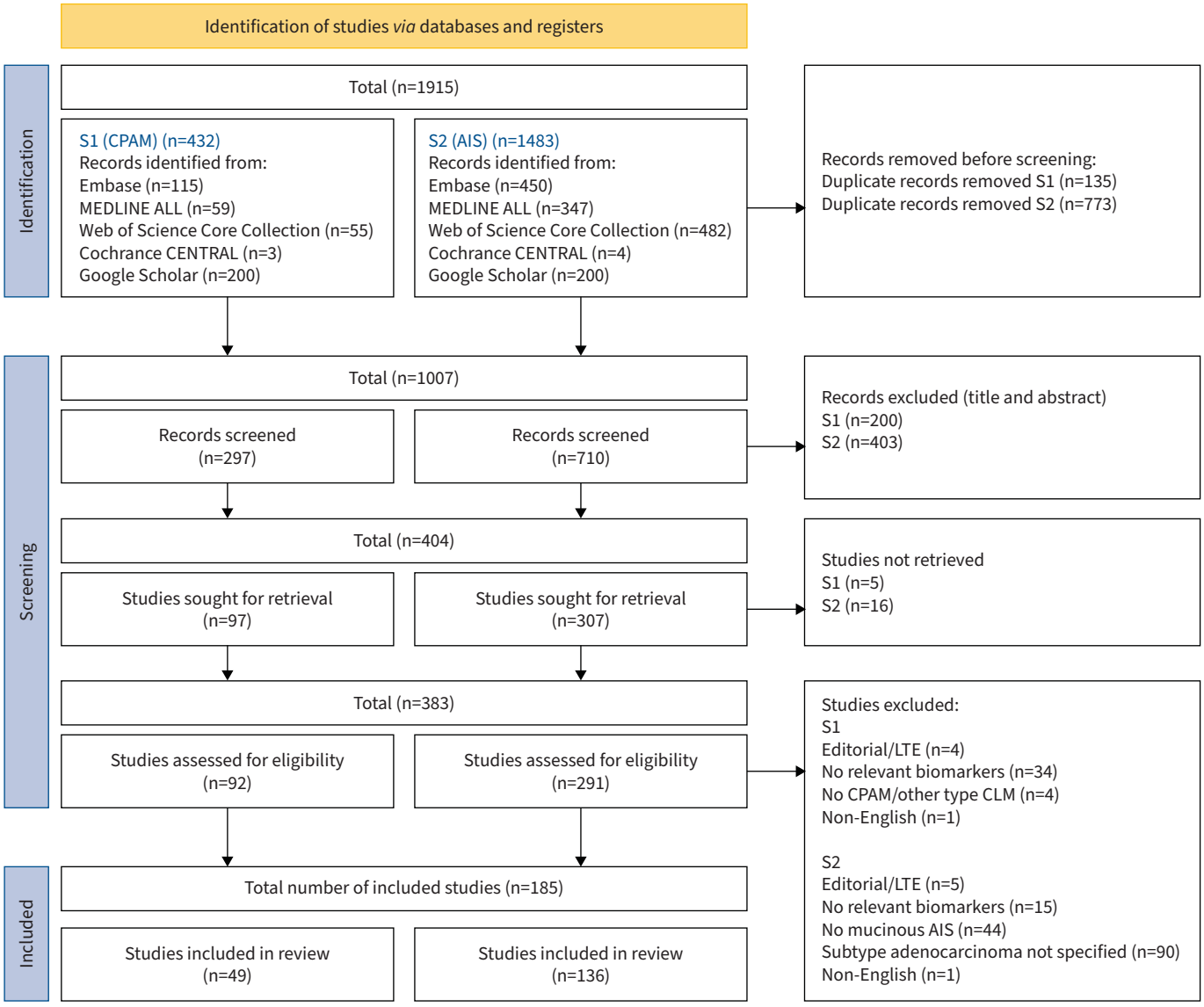

FIGURE 2 Preferred Reporting Items for Systematic reviews and Meta-Analyses flowchart. CPAM: congenital pulmonary airway malformation; AIS: adenocarcinoma *in situ*; LTE: letter to the editor; CLM: congenital lung malformation.

Data collection process and data items

A data extraction form was designed, using Microsoft Excel. The following data were extracted from every included article: author, year of publication, country of origin, study design and characteristics of the target. Relevant outcome measures were 1) type of target (name, localisation, type); 2) upregulation or downregulation of the target in CPAM tissue, or in AIS compared to healthy lung tissue and its relationship to the KRAS pathway; and 3) presence of symptoms in CPAM patients. Data charting was performed by one investigator (M.J.P. Zuidweg) and checked by a second investigator (C. van Horik). This check showed no discrepancies.

Critical appraisal of evidence

A critical appraisal of individual sources of evidence was not performed. This review aimed to provide an overview of the extent and nature of all existing evidence concerning our topic; therefore, a critical appraisal tool was undesirable [34, 35].

Selection of potential targets

Out of the selected articles from each separate search string, all the targets were described, emphasising the membrane-bound targets. Targets in CPAM tissue were compared with targets in AIS. If the target was

directly or indirectly related to the KRAS pathway, this relationship was described. The relationship of the found proteins to the KRAS pathway was determined, using the Kyoto Encyclopedia of Genes and Genomes. For confirmation of a target's association to the plasma membrane, information from GeneCards and The Human Protein Atlas was used. The RNA expression of interesting targets was evaluated in the LungMAP Single Cell Reference v1 web portal [36].

Haematoxylin and eosin staining and immunofluorescent staining

The CPAM tissue sections were gathered as described by Hermelijn *et al.* [24] from cases diagnosed between January 1990 and January 2019.

Sections were stained with haematoxylin and eosin (H&E). Pictures were made using a bright-field microscope (Olympus BX41).

For immunofluorescent staining the 5-µm sections adjacent to the used H&E-stained sections were used. The sections were deparaffinised, rehydrated and washed. Antigen retrieval with Tris-EDTA buffer (10 M Tris, 1 M EDTA, pH 9.0) was used. Indirect immunofluorescent staining was performed. Primary antibodies used for immunofluorescent staining were MUC1 (AMAb191533, mouse, 1:100; Atlas antibodies), SOX2 (14-9811-82, rat, 1:800; ThermoFisher), CDH1 (sc-8426, mouse, 1:500; Santa Cruz), ERBB2 (4290, rabbit 1:250; Cell signalling), CEACAM5 (7072, mouse, 1:50; Dako). The following secondary antibodies were used: donkey anti-mouse with AF 488 (715-545-151, 1:500; Jackson ImmunoResearch), donkey anti-rat with AF 594 (712-585-153, 1:500; Jackson ImmunoResearch), donkey anti-rabbit 594 (711-585-152; Jackson ImmunoResearch) and 4′,6-diamidino-2-phenylindole (564907, 1:2000; BD Pharmingen). All sections were imaged on a confocal microscope (STELLARIS 5; Leica Microsystems). Images were evaluated by C. van Horik, R.J. Rottier, W.S.F.J. Tummers and J.M. Schnater.

Results

To address the research question, two distinct systematic literature searches were performed, as outlined in the methodology section. The initial search (S1) aimed to identify all potential targets in CPAM patients, while the second search (S2) focused on identifying potential targets in AIS patients. Articles with the most potential to include relevant targets, as indicated by their abstracts, were comprehensively reviewed. In the two searches combined 1915 articles were identified (S1 n=432, S2 n=1483). The details of the article selection are shown in figure 2. After removing duplicates and screening on title and abstract, 383 remained (S1 n=92, S2 n=291). These articles were assessed for eligibility based on the inclusion and exclusion criteria as described in the Methods section. Eventually, 185 articles were included (S1 n=49, S2 n=136). These include 130 comparative studies (S1 n=18, S2 n=112), 30 case studies (S1 n=24, S2 n=6), 25 reviews (S1 n=6, S2 n=19) and one "brief communication" (S1).

This study elaborates on specific targets in CPAM and their potential use in targeted molecular imaging. Therefore, targets with specific aspects such as upregulated expression through the CPAM tissue compared to expression of normal lung (or $KRAS^+$ CPAM compared to $KRAS^-$ CPAM) tissue, association with the plasma membrane [23, 37] and expression in AIS where assessed.

Tested targets in tissue from CPAM patients

In total, 143 possible targets were described in the articles from S1. From all these targets, 57 were described as not altered from control (if they were compared to control tissue) or not upregulated. For a complete overview of all targets tested in CPAM, but not differentially expressed or not mutated targets, the type of protein and the described expression are listed in supplementary table S1. Downregulated targets can also be used in targeted molecular imaging, but are more challenging. Therefore, all four targets described as downregulated in CPAM patients are listed in supplementary table S2. From the resulting 82 targets, 62 were not localised in the plasma membrane. The remaining, not plasma membrane localised, upregulated targets are described in supplementary table S3.

From all 143 potential targets, the first selection of upregulated and membrane-bound proteins (or genes coding for membrane-bound proteins) resulted in 20 targets. These 20 targets include 11 receptors, five glycoproteins, one cell adhesion molecule, one signalling cytokine, one hydrolase and one aminopeptidase. These targets, the type of protein and the described expression are described in supplementary table S4. This selection process for most interesting targets is summarised in figure 3.

Overlapping cell surface targets upregulated in both CPAM tissue and AIS tissue

To stratify CPAM patients with molecular imaging into high- and low-risk groups for developing malignancy, we hypothesised that targets that were upregulated in both CPAM tissue and AIS tissue could

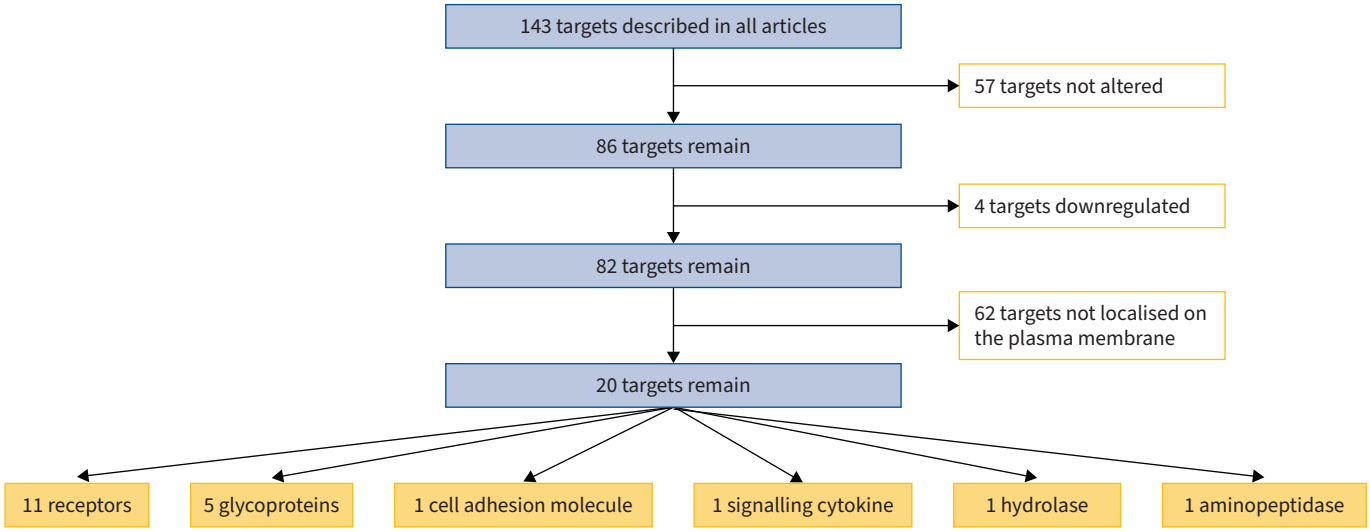

FIGURE 3 Flowchart describing the congenital pulmonary airway malformation target selection process.

be more promising. The resulting 20 targets were compared to the targets described in AIS, found in S2. Out of the 20 cell surface targets found in CPAM, six targets were also expressed or upregulated in AIS: CEA cell adhesion molecule 5 (CEACAM5 or CEA), E-cadherin (CDH1), epidermal growth factor receptor (EGFR), Erb-B2 receptor tyrosine kinase 2 (ERBB2), integrin-α2 (ITGA2) and mucin 1 (MUC1). These targets, their location and their expression in CPAM and AIS (or if tested in adenocarcinoma and atypical adenomatous hyperplasia) with the corresponding articles, are described in table 1.

CAECAM5 was scarcely studied in CPAM patients, in which two out of three patients already harboured malignant cells, and one of them found no expression in the CPAM tissue itself [38–40]. In AIS, the RNA expression and protein expression of CEACAM5 was described as high in five studies [41–45].

E-cadherin was not studied extensively in both CPAM and AIS. In CPAM, Volpe *et al.* [46] described upregulation of the protein by Western blotting. In AIS, two out of three studies described upregulation of RNA expression or protein expression [47, 48], while Nakagiri *et al.* [49] described only rare expression of E-cadherin in AIS. EGFR mutations were described in AIS, and only one documented CPAM case report of an 80-year-old patient with a CPAM with ADC [40]. However, the EGFR protein was expressed in both CPAM and AIS. EGFR was described to be more expressed in highly invasive malignancies than in AIS; however, they did not describe to what extent it was expressed in normal lung tissue [50–58]. ERBB2 is more highly expressed in CPAM tissue and is even higher in mucinous proliferations than normal lung tissue according to Fakler *et al.* [59]; Rossi *et al.* [51] and Kim *et al.* [60] describe expression only in mucinous proliferations. In AIS tissue, the expression was higher compared to normal bronchial epithelium. ITGA2 is scarcely studied in CPAM and AIS, but the two studies showed high expression, and interestingly, Volpe *et al.* [46] found a high expression of the membrane-bound domain of integrin-α2. MUC1 seems to be progressively upregulated during proliferation of tissue, although the studies described do contradict each other on this matter [43, 64, 65].

Overlapping cell surface targets upregulated in CPAM and AIS with an association to the KRAS pathway

From the six targets upregulated in both CPAM and AIS tissue, five targets could be found in the KRAS (or a KRAS-associated) pathway (figure 4).

Validation of potential targets

Next, we evaluated the identified targets for their potential use as target for molecular imaging. The expression would be preferably found in bronchial epithelial cell types, as CPAM cyst linings consist of bronchial epithelial cells [66]. Therefore, the expression pattern of these targets was analysed using existing single-cell RNA sequencing data (figure 5) [36]. *EGFR* was extensively found in a variety of cells, and not clearly overexpressed in one cell type. For the epithelial cells it was mostly expressed in alveolar type 1 and 2 cells and a small group of basal/suprabasal cells. Besides, it was also found in

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TABLE 1 Expression of targets described as upregulated in both congenital pulmonary airway malformations (CPAM) and adenocarcinoma *in situ* (AIS)

Protein (or gene) name; target type	Subcellular location	CPAM /AIS	Described expression	References
CEA cell adhesion molecule 5 (*CEACAM5* gene); glycoprotein	Plasma membrane (or serum)	CPAM	Three case reports (n=3) Benouaich *et al.* [38]: CPAM 1 with mixed ADC (77-year-old patient), positive IHC Li *et al.* [39]: CPAM 1 with BAC (2-day-old patient), positive IHC Hasegawa *et al.* [40]: CPAM 1 with ADC (80-year-old patient), positive IHC (and negative in surrounding control tissue)	[38–40]
		AIS	Four comparative studies (n=93), one review Jian *et al.* [41]: (6 out of 10 patients), positive IHC Tone *et al.* [42]: AIS and ADC (63 patients), upregulated RNA in RNA expression Maeshima *et al.* [43]: (17 out of 20 patients), positive IHC Barekman *et al.* [44]: (20 patients), positive IHC with sensitivity 50%, specificity 76% Mori *et al.* [45]: AAH-positive IHC (higher intensity in ADC)	[41–45]
E-cadherin (*CDH1* gene); cell adhesion molecule	Plasma membrane, Golgi apparatus	CPAM	One comparative study (n=7) Volpe *et al.* [46]: upregulated in Western blot	[46]
		AIS	Three comparative studies (n=158) Goodwin *et al.* [47]: (3 patients) upregulated RNA in RNA-sequencing Kerr *et al.* [48]: (36 out of 107 AAH patients, 12 out of 18 AIS patients), positive IHC Nakagiri *et al.* [49]: (30 patients), rare expression IHC	[47–49]
Epidermal growth factor receptor (*EGFR* gene); receptor (tyrosine kinase)	Plasma membrane	CPAM	Two case studies (n=64), one comparative study (n=5), one case report Hasegawa *et al.* [40]: CPAM 1 with ADC (80-year-old patient) gene mutation Guo *et al.* [50]: (23 patients) positive IHC, but no expression in mucinous proliferations Rossi *et al.* [51]: (40 out of 41 patients), positive IHC, no EGFR gene mutation Hong *et al.* [52]: (4 patients), upregulated RNA in RNA-sequencing and upregulated mRNA in qRT-PCR	[40, 50–52]
		AIS	35 comparative studies, two case reports, 13 reviews All studies describe either or both RNA levels with PCR and positive IHC expression in AIS tissue, or describe genetic mutations in the *EGFR* gene	[53–58]
Erb-B2 tyrosine kinase 2 (*ERBB2* gene); receptor (tyrosine kinase)	Plasma membrane	CPAM	One case report, two comparative studies (n=69) Fakler *et al.* [59]: (28 patients), positive IHC in CPAM cyst and more expressed in mucinous proliferations and ADC Rossi *et al.* [51]: (40 out of 41 patients), IHC only positive in mucinous proliferations, no *HER2* mutations Kim *et al.* [60]: (1 patient), IHC only positive in mucinous proliferations	[51, 59, 60]
		AIS	Three comparative studies (n=133) Saad *et al.* [61]: (9 out of 50 patients), positive IHC (19 out of 50 in ADC) Zhong *et al.* [58]: (0 out of 60 patients), negative IHC (higher expression by IHC and Western blot in ADC) Xu *et al.* [62]: (1 out of 18 AAH, 3 out of 28 AIS), gene mutations with NGS	[58, 61, 62]

Continued

TABLE 1 Continued

Protein (or gene) name; target type	Subcellular location	CPAM /AIS	Described expression	References
Integrin-α2 (*ITGA2* gene); receptor	Plasma membrane, nucleoplasm	CPAM	One comparative study (n=7) Volpe *et al.* [46]: (7 patients), upregulated in Western blot and IHC (for extracellular antibody)	[46]
		AIS	One comparative study (4 cell lines) Guo *et al.* [63]: (4 cell lines), expressed in microarray, not validated further	[63]
Mucin 1 (*MUC1* gene); glycoprotein	Plasma membrane	CPAM	One case report, one comparative study (n=42) Rossi *et al.* [51]: (12 out of 41 patients) IHC moderate expression, but no expression in mucinous proliferations Kim *et al.* [60]: (1 patient), IHC CPAM cyst positive, but negative in mucinous proliferations	[51, 60]
		AIS	Three comparative studies (n=51) Maeshima *et al.* [43]: (13 out of 20), positive IHC Awaya *et al.* [64]: (27 patients) 15 low, 12 high IHC expression in AIS and (11 low and 47 high out of 58 patients) in AAH; less expression in more advanced malignancies Copin *et al.* [65]: moderate IHC in AIS (in type II pneumocyte hyperplasia) by ISH	[43, 64, 65]

ADC: adenocarcinoma; IHC: immunohistochemistry; BAC: bronchioalveolar carcinoma; AAH: atypical adenomatous hyperplasia; qRT-PCR: real-time quantitative reverse transcription PCR; NGS: next-generation sequencing; ISH: *in situ* hybridisation.

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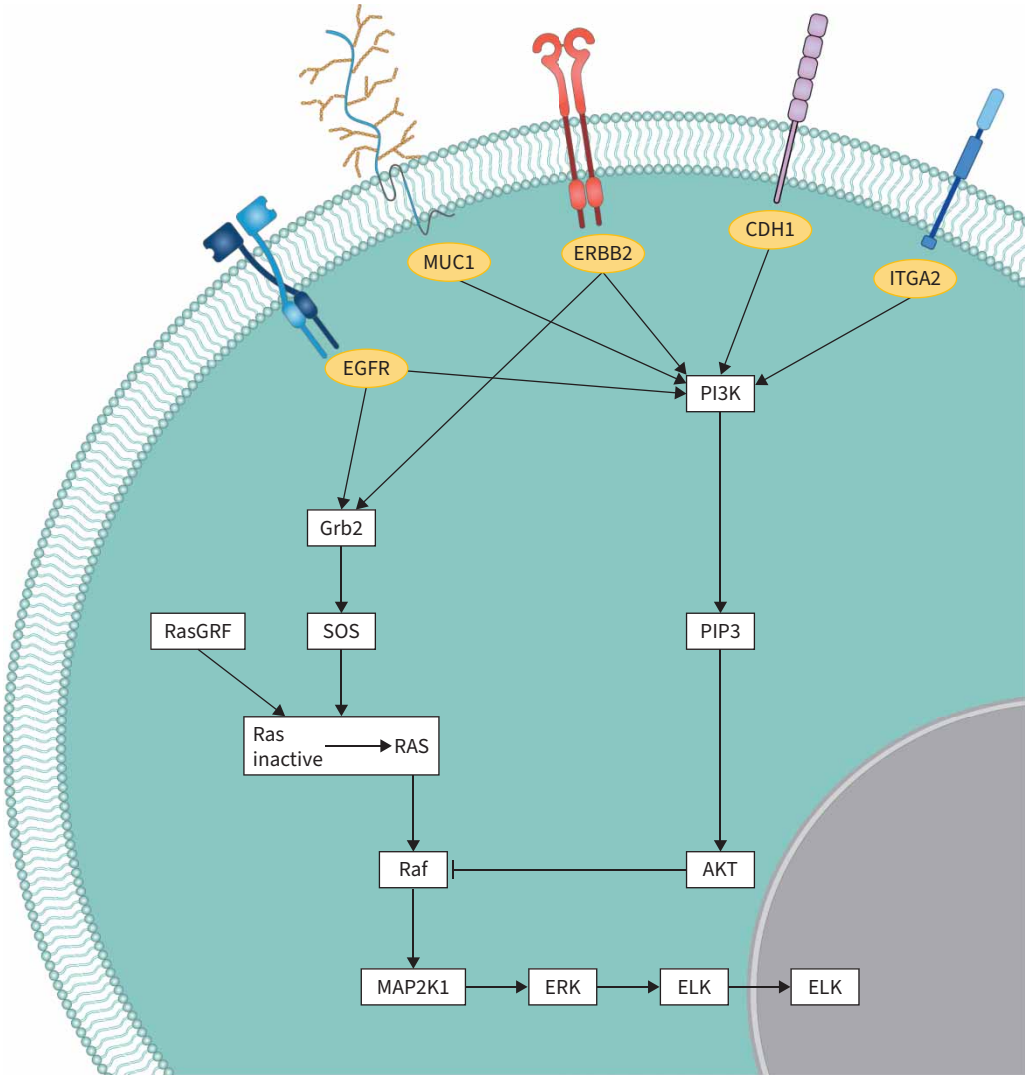

FIGURE 4 KRAS-related targets. Upregulated targets related to the RAS pathway: all potential plasma membrane targets related to the RAS pathway found in the literature search (therefore CEACAM5 is not shown). EGFR: epidermal growth factor receptor; MUC1: mucin 1; ERBB2: Erb-B2 tyrosine kinase 2; CDH1: E-cadherin; ITGA2: integrin-α2; →: activation; ⊥: inhibition.

mesenchymal cell types. *ITGA2* was highly expressed in alveolar fibroblasts, a population of mesenchymal cells and alveolar type 1 cells express *ITGA2* mildly. CDH1 seems to be more specific for epithelial cell types compared to *EGFR* and *ITGA2*. It was expressed in alveolar type 1 and 2 cells, ciliated cells, respiratory airway secretory cells and also mildly in basal/suprabasal cells. The expression of *ERBB2* is comparable with the expression of *EGFR*; it was found in the same epithelial and mesenchymal cell types, but mostly the expression was lower. However, in contrast to *EGFR*, the expression of *ERBB2* was also found in natural killer cells. *CEACAM5* was quite specifically overexpressed in three epithelial cell types; alveolar type 2 cells and goblet cells, and a part of the basal/suprabasal cells. *MUC1* was expressed by several epithelial cell types; alveolar type 1 and 2 cells, secretory cells and goblet cells, and to a small extent by basal/suprabasal cells.

Because EGFR and ITGA2 show high expression in other cell types than epithelial cells, they are not suitable as a target. The other four targets show high expression in one or more epithelial cell types, and therefore deserve further validation. In order to map the spatial expression of CDH1, CEACAM5, ERBB2 and MUC1, immunofluorescence was performed on CPAM tissue with KRAS mutations ($KRAS^{+}$) and CPAM tissue without KRAS mutations ($KRAS^{-}$) and the adjacent normal lung tissue.

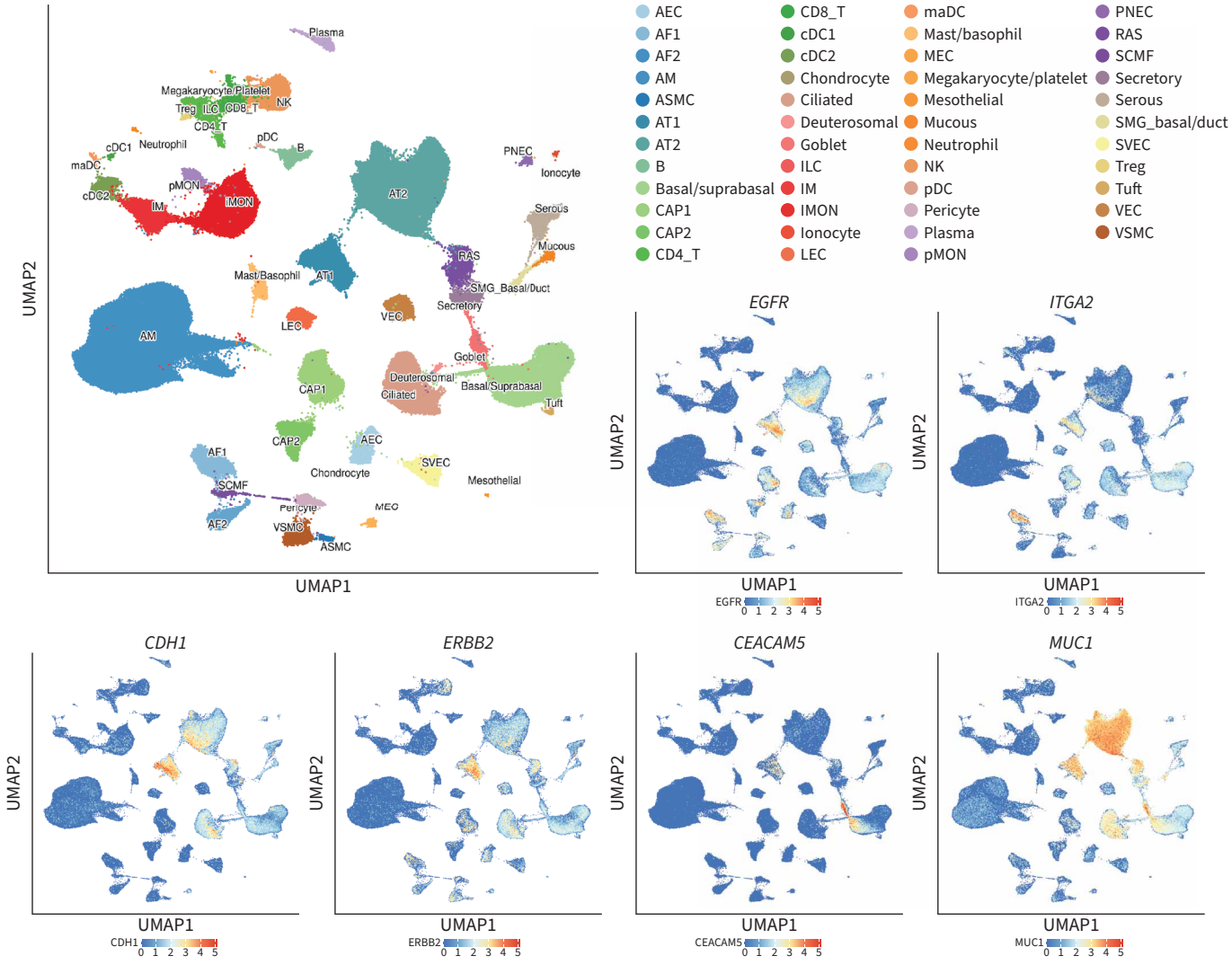

FIGURE 5 Single-cell RNA expression of pulmonary cell types according to Guo *et al.* [36] and the RNA expression of our selected interesting targets in these cells. Red indicates high RNA expression of the target, blue indicates low expression of the target. AEC: arterial endothelial cell; AF: alveolar fibroblast; AM: alveolar macrophage; ASMC: airway smooth muscle cell; AT: alveolar type cell; CAP: capillary cell; ILC: innate lymphoid cell; IM: interstitial macrophage; IMON: inflammatory monocyte; LEC: lymphatic endothelial cell; maDC: mature dendritic cell subset; MEC: myoepithelial cell; NK: natural killer cell; pDC: plasmacytoid dendritic cell; pMON: patrolling monocyte; PNEC: pulmonary neuroendocrine cell; SCMF: secondary crest myofibroblast; SMG: submucosal gland; VEC: venous endothelial cell; VSMC: vascular smooth muscle cell.

A small-scale staining to explore these markers is shown in figure 6. CDH1 was broadly expressed in all epithelial cells (figure 6a). The expression and staining pattern were similar in all tissues. ERBB2 was expressed in all bronchial epithelial cells in the adjacent lung tissue, but not in the KRAS^{-} CPAM tissue. There was expression of ERBB2 in the epithelial cells in the KRAS^{+} CPAM tissue, but in fewer cells than in the bronchial epithelium of the adjacent lung tissue. CEACAM5 was only expressed in a limited number of cells (arrows in figure 6c), roughly in the same numbers of cells in all tissues.

MUC1 strongly positive in mucinous proliferations

The expression analysis showed that MUC1 could be a promising target for imaging. Therefore, MUC1 expression was analysed in lung tissue of another seven KRAS^{-} CPAM and nine KRAS^{+} CPAM patients. All nine KRAS^{+} CPAM tissues contained mucinous proliferations [24]. H&E staining confirmed the presence of CPAM characteristic tissue (figure 7a); however, from the nine KRAS^{+} CPAM tissues analysed, mucinous proliferations were found only in five of these new sections (figure 7a).

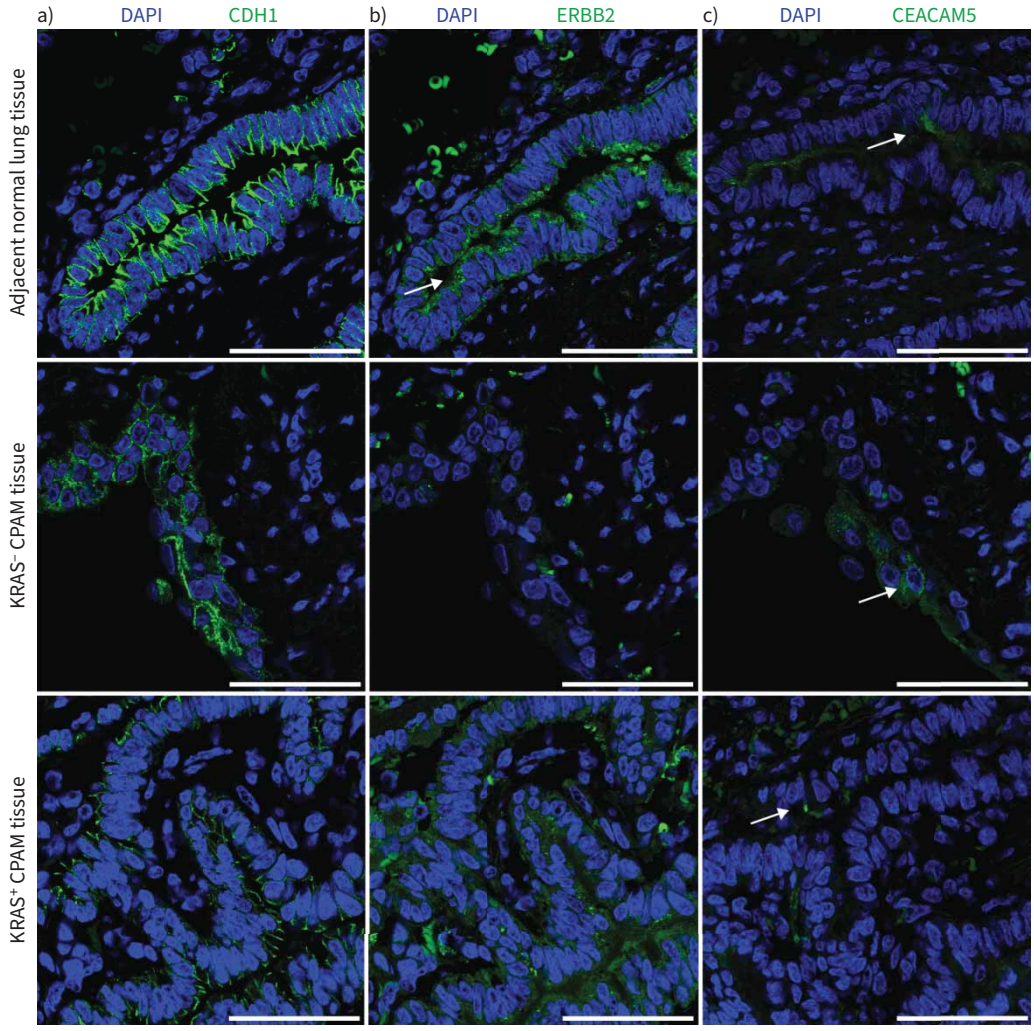

FIGURE 6 Immunofluorescent staining of adjacent "normal lung" tissue, $KRAS^-$ congenital pulmonary airway malformation tissue (CPAM) and $KRAS^+$ CPAM tissue with a) E-cadherin (CDH1), b) Erb-B2 receptor tyrosine kinase 2 (ERBB2) and c) cell adhesion molecule 5 (CEACAM5). DAPI: 4′,6-diamidino-2-phenylindole. Arrows point to positive signal. Scale bars=50 µm.

Immunofluorescence staining was performed for MUC1 and the general airway epithelial marker SOX2 [67]. As shown in figure 7b, SOX2 is expressed in the epithelial lining of the CPAM, both $KRAS^+$ and $KRAS^-$, and also in the bronchial epithelium of the adjacent normal lung tissue. Only a few $MUC1^+$ cells are present in the normal lung tissue adjacent to the cystic lesion, and in the $KRAS^-$ and $KRAS^+$ CPAM tissue regions without the mucinous proliferations (arrows in figure 7b). However, MUC1 was highly expressed in four out of five the mucinous proliferations of the $KRAS^+$ CPAM samples (figure 7b and 7c). In the fourth picture from figure 7c the MUC1 staining did not show clear positivity of MUC1 in the mucinous proliferative cells. However, the mucinous proliferations clusters are quite small, and the mucinous cells in this fourth sample might be disrupted. There were no other mucinous proliferation clusters found in the remaining sections of this sample.

Current pre-clinical ligands for MUC1

The development of a targeting ligand is an extensive process. Therefore, the existing targeting ligands where assessed in literature. MUC1 is a membrane bound mucin, consisting of an N-terminal domain and a C-terminal domain. The extracellular N-terminal domain contains 20-amino acid tandem repeats (VNTR) and a sperm protein-enterokinase-agarin domain, while the C-terminal domain has a short extracellular domain, a transmembrane domain and a cytoplasmic tail [68]. The N-terminal domain is extensively glycosylated, which is affected in various types of cancer [69].

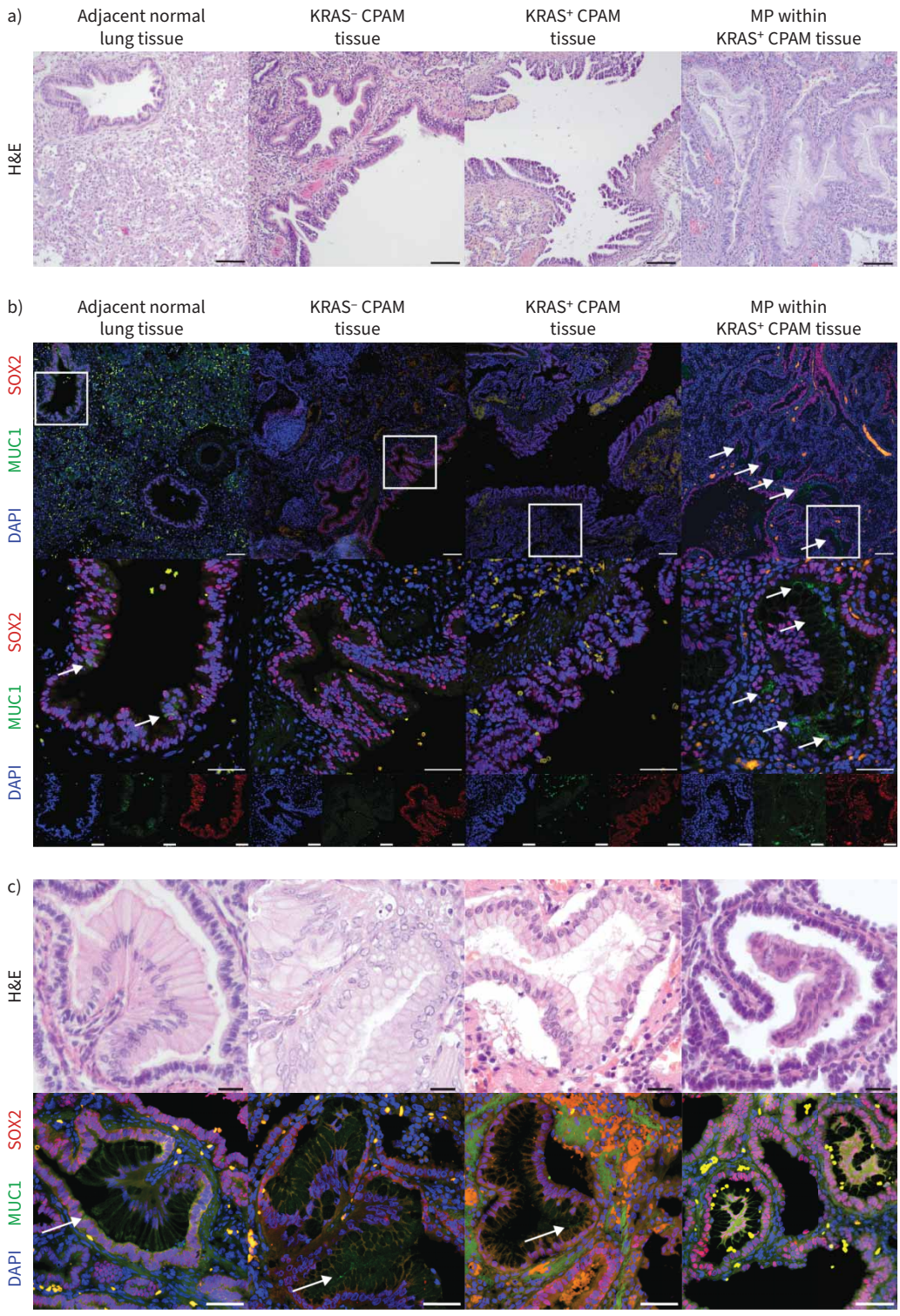

FIGURE 7 a) Haematoxylin and eosin (H&E) staining of adjacent "normal" lung tissue, congenital pulmonary airway malformation (CPAM) tissue without KRAS mutations ($KRAS^-$), CPAM tissue with KRAS mutations ($KRAS^+$) and areas with mucinous proliferations (MP) found in only $KRAS^+$ CPAM tissue. Scale bars=100 µm. b) Immunofluorescent staining of $KRAS^-$ CPAM tissue, $KRAS^+$ CPAM tissue and MP within $KRAS^+$ CPAM tissue with mucin 1 (MUC1), sex-determining region Y-box 2 (SOX2) and 4′,6-diamidino-2-phenylindole (DAPI). Scale bars=100 µm (top row), 50 µm (bottom rows). c) H&E staining of MPs, and immunofluorescent staining of MUC1, SOX2 and DAPI. Scale bars=50 µm. Arrows point to positive signals.

So far, numerous targeted agents are developed specifically for different domains of MUC1; the VNTR domain, the extracellular part of the C-terminal domain and the cytoplasmic tail [70, 71]. Because in most cancers cells MUC1 is hypoglycosylated and thereby the VNTR domain is exposed, multiple targeted agents are developed for this specific domain. Six targeted agents are currently in clinical trials (huC242, huPAM4, hPAM4 (clivatuzumab), SAR56665 8huDS6-DM4, PankoMab-GEX, a PD-1 inhibitor armed with an anti-MUC1 (PankoMab), and an anti-CD3 bispecific antibody, AR20.5) [70]. All of these targeted agents bind to the VNTR domain of MUC1. However, none of these targeted agents have yet been clinically approved. Current antibodies fail due to shedding of the VNTR epitope into the circulation [72]. Therefore, Maleki *et al.* [72] propose using the MUC1-C terminal domain, and since we identified an antibody that recognised this domain, it could be of potential use for stratifying CPAM patients.

Discussion

This study aimed to provide an overview of targets that could be of importance for the development of targeted molecular imaging to stratify CPAM patients into low- and high-risk groups for the probability of developing malignancy later in life and to validate the resulting promising targets. Therefore, all upregulated membrane-bound targets in both CPAM and AIS tissue were described and the first step towards validation of an interesting target was accomplished. The systematic search resulted in six possible targets that have potential in stratifying CPAM patients into low- and high-risk groups: CEACAM5, E-cadherin, EGFR, ERBB2, ITGA2 and MUC1. E-cadherin, ERBB2 and CEACAM5 did not differ between adjacent lung tissue and both $KRAS^+$ and $KRAS^-$ CPAM tissue. MUC1 was found to be positive in most mucinous proliferations, a precursor for the AIS that could develop in CPAM patients.

We hypothesised that KRAS would be an important starting point to discriminate between high- and low-risk CPAM patients. In other types of cancer, KRAS mutations seem to be the main cause of malignant transformation. For example, KRAS mutations are the key initiator of pancreatic cancer [73]. In nonsmall cell lung cancer, KRAS is also considered to be an oncogenic driver [74]. Many articles that were included did describe KRAS mutations in CPAM and/or AIS tissue as well. Unfortunately, being located on the inside of the cell, KRAS is not yet suitable for targeting in molecular imaging.

Another gene, frequently associated with malignant progression of CPAM is DICER1. DICER1 is associated with CPAM type 4 and pleuropulmonary blastoma in particular [75]. However, we only identified this gene twice in our search. This is probably due to the fact that DICER1 is associated with CPAM type 4 [75, 76] and KRAS is mostly associated with CPAM type 1 and type 2 [17, 27, 28, 29–31]. Although this should be taken into consideration, DICER1 mutations appear to be less relevant for this purpose since these mutations are less common in CPAM (and AIS) compared to KRAS mutations. Also, like KRAS, DICER1 is not located on the plasma membrane and therefore not suitable as a possible target for molecular imaging [77].

This study holds some limitations. Some of the targets are only scarcely tested and results, extracted from articles with small sample sizes, can potentially be less reliable. This should be taken into account when valuing the possible targets. Furthermore, several targets were found by testing DNA or RNA expression in the tissue. Those targets were not validated at the protein level. It is therefore uncertain whether these targets itself are upregulated in CPAM or AIS tissue. Moreover, some studies did not compare the CPAM tissue or AIS tissue with healthy lung tissue. Furthermore, we described only targets already described in the literature. Therefore, it is possible that potential promising targets are missed to distinguish between high- and low-risk CPAM patients. Lastly, for the verification of the targets, only a limited amount of CPAM tissue was available.

Our most promising target, MUC1, was only detected in mucinous proliferations and did not specifically differentiate between $KRAS^+$ and $KRAS^-$ CPAM tissue itself. Thus, the $KRAS^+$ patients without mucinous proliferations could not be selected with MUC1. However, we showed that all CPAMs with mucinous proliferations are $KRAS^+$ [24] and because mucinous proliferation is a precursor for AIS we propose that targeting mucinous proliferations could suffice in stratifying CPAM patients into high and low risk of developing malignancy. Besides, MUC1 expression was described in AIS and progressively upregulated in more invasive lung carcinomas [78]. In conclusion, MUC1 is a potentially interesting target for imaging.

Future research should focus on further verifying MUC1 as a target that can distinguish between CPAM patients with high and low risk of developing malignancy. Unfortunately, no mouse model has so far been developed for CPAM. However, we are able to successfully culture airway organoids and air–liquid interface cultures derived from CPAM cyst tissue. With these cultures it is possible to verify differences in

MUC1 expression in $KRAS^+$ and $KRAS^-$ CPAM tissue. Additionally, it is important to verify whether MUC1 in CPAM is hypoglycosylated in CPAM patients. This is importing to assess which type of targeting ligand should be developed. An approach to develop a targeting ligand is the use of aptamers (RNA or DNA oligomers generated by the combinatorial Evolution of Ligands by Exponential methodology), because they bind to a desired targeted with a high affinity and specificity and are capable of carrying a radionuclide [79]. In addition, peptides (small biomolecules) can carry radiolabels and can be developed for a fast distribution in target tissue, rapid blood clearance and high tumour-to-background ratios, and therefore might also be of use for developing a ligand [80, 81].

Despite the limitations, this study provides an extensive overview of all known targets in CPAM and their expression in AIS and is an important first step in identifying a target that might stratify CPAM patients. In conclusion, the aim of this article was to stratify asymptomatic CPAM patients into high- and low-risk group for developing malignancy. The current hypothesis is that mucinous proliferations develop into adenocarcinoma *in situ*. A noninvasive method that could detect these mucinous proliferations could help in the clinical decision-making towards surgery; in that case, only the asymptomatic CPAM patients harbouring a mucinous proliferation could be operated.

Points for clinical practice

- MUC1 is a potential target for stratifying CPAM patients into a high- and low-risk groups for developing malignancy. If further verified, this target should select patients at high risk of developing malignancy and help surgeons to select only the MUC1-positive (asymptomatic) patients for surgical resection.

Questions for future research

- Is MUC1 hypoglycosylated in mucinous proliferations in CPAM tissue?
- Could further analysing patient-derived material from CPAM patients, such as organoid cultures and air–liquid interface cultures further verify that MUC1 is a potential target in stratifying CPAM patients?
- Can we develop a working targeting ligand for MUC1 that can stratify CPAM patients in targeted molecular imaging?

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