



## BASIC SCIENCE ARTICLE

# CD44 and RHAMM expression patterns in the human developing lung

Laszlo Markasz<sup>1</sup>, Rashmin C. Savani<sup>2</sup>, Anders Jonzon<sup>1</sup> and Richard Sindelar<sup>1</sup>

**BACKGROUND:** The hyaluronan (HA) receptors CD44 and RHAMM (CD168) are involved in cellular proliferation, differentiation, and motility. As previously investigated, HA and RHAMM expression in human neonatal lungs correlates to gestational age (GA) and air content.

**METHODS:** CD44 immunofluorescence was analyzed in postmortem lung samples from infants ( $n = 93$ ; 22–41 GA) by digital image analysis together with clinical data, including RHAMM expression, lung air, and HA content by hierarchical clustering.

**RESULTS:** Five groups were defined according to RHAMM/CD44 expression, GA, and postnatal age (PNA): extremely to very preterm (EVP; 22–31 GA; Groups 1–2), moderately preterm to term (MPT; 31–41 GA; Groups 3–4), and mixed preterm to term (27–40 GA; Group 5). CD44 correlated linearly with RHAMM in MPT ( $r = 0.600$ ;  $p < 0.004$ ). In EVP, high CD44 and low RHAMM corresponded with high PNA and lung air content independently of HA and GA (Group 1 vs 2;  $p < 0.05$ ). In MPT, high and low CD44 corresponded with low and high RHAMM independently of GA, HA, and lung air content (Group 3 vs 4;  $p < 0.001$ ). No correlation between CD44 and GA/PNA at death was observed.

**CONCLUSIONS:** A linear correlation between CD44 and RHAMM expression occurs during the late saccular phase of lung development at birth, whereas postnatal influences on CD44 and RHAMM expression in extremely to very preterm infants cannot be excluded.

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**IMPACT:**

- The interplay between CD44 and RHAMM, two receptors of hyaluronic acid, can be dependent on the lung developmental stage at birth.
- This is the second study that analyzes the distribution pattern of CD44 in the human lung during development and the first study performed with quantitative analysis of CD44 expression together with RHAMM expression in the human lung.
- Our results suggest a relationship in a subset of infants between CD44 and RHAMM expression, which appears at birth during the late saccular stage but not during the earlier stages of lung development.

**INTRODUCTION**

The mechanisms leading to disease in the developing lung involve numerous signaling pathways.<sup>1</sup> As part of the extracellular matrix (ECM), hyaluronan (HA) plays an important role in inflammation and tumorigenesis and also in developmental processes.<sup>2</sup>

CD44 and receptors for HA-mediated motility (RHAMM, CD168) are two major receptors for HA involvement in cell proliferation, cell differentiation, and motility.<sup>3</sup> There are indications that both CD44 and RHAMM are involved in the pathophysiology of the lung.<sup>4,5</sup> CD44 and RHAMM have different functions that control proliferation and migration.<sup>6</sup> The functions of CD44 are less specific compared to RHAMM. Numerous studies have shown that CD44 can bind to other glycosaminoglycans,<sup>7</sup> mucosal addressin,<sup>8</sup> collagen type I,<sup>9</sup> and fibronectin.<sup>10</sup>

Alternative splicing and/or posttranslational modification generates multiple CD44 isoforms,<sup>11</sup> which distribute differentially in

the lung. The standard form of CD44 is present at the cell surface of alveolar macrophages, in some interstitial cells, and in epithelial cells; while the variant form of CD44 (CD44v) is mostly localized to epithelia.<sup>4</sup> Kasper et al. showed that CD44 is present during the embryonic stage of lung development (10 weeks' gestational age [GA]) and confined to the developing epithelium and some interstitial cells in the stem bronchus and the bronchial bud.<sup>4</sup> During the pseudoglandular and canalicular stages, CD44 expression appears as a lateral staining on the epithelial cell membranes, and alveolar macrophages show a strong staining intensity (studied at 12–21 weeks' GA).<sup>4</sup> At the saccular stage, a more cytoplasmic expression pattern in bronchial epithelial cells could be detected (studied at 34 weeks' GA),<sup>4</sup> and this pattern remains in normal adult lung tissue.<sup>12</sup> Ambalavanan et al. showed that CD44 mRNA decreases during alveolar septation in mice and increases during hyperoxia exposure or the development of bronchopulmonary dysplasia.<sup>13</sup>

<sup>1</sup>Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden and <sup>2</sup>Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX, USA

Correspondence: Laszlo Markasz (laszlo.markasz@kbh.uu.se)

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CD44 regulates macrophage recruitment to the lung,<sup>14</sup> whereas the surfactant protein-A-stimulated chemotaxis of macrophages is mediated by RHAMM and HA but not by CD44.<sup>15</sup> Alveolar macrophages express high levels of CD44 and are one of the few macrophage populations that can constitutively bind HA.<sup>16</sup> CD44 is responsible not only for the binding but also the uptake of HA in alveolar macrophages in vitro and during lung development after birth.<sup>17</sup> CD44 also has an important role in both the acute and late inflammatory response in the lung.<sup>18</sup> Infectious and noninfectious models of lung injury suggest that CD44 can modify inflammatory cell recruitment to the lung via ligation to HA,<sup>19</sup> act as a negative regulator of acute pulmonary inflammation,<sup>18</sup> and aid in resolving lung inflammation.<sup>19</sup> In addition, CD44-deficient mice show increased mortality and unremitting inflammation in a mouse model of bleomycin-induced lung injury.<sup>19</sup>

The effects of CD44 signaling on cell behavior is controversial. In mouse models, CD44 is protective during hyperoxia-induced lung injury,<sup>20</sup> and lung mesenchymal stem cells expressing CD44 can inhibit the proliferation of fibroblasts and enhance epithelial repair in vitro.<sup>21</sup> However, Li et al. observed that severe lung fibrosis in adults requires an invasive fibroblast phenotype regulated by HA and CD44.<sup>22</sup>

The biological functions of CD44 have been investigated most comprehensively in human cancer,<sup>11,23</sup> but the role of CD44 in fetal and postnatal lung development is much less described. Most of the existing studies have been performed in animal models or under in vitro conditions.<sup>22,24</sup> There are only a few works published on CD44 expression in the fetal human lung,<sup>4,25</sup> and these studies lack quantifying techniques.

In our previous work, we investigated RHAMM expression compared to HA concentration and lung air content in neonates.<sup>26</sup> The aim of the present work was to study CD44 expression related to RHAMM expression, as well as lung air and HA content in the human neonatal lung through different developmental stages and postnatal ages (PNAs) by using semiquantitative digital techniques and hierarchical cluster analyses. Based on animal studies,<sup>13,14</sup> we hypothesized that CD44 and RHAMM can influence each other due to their involvement in HA homeostasis.

## MATERIAL AND METHODS

### Lung tissue samples

The tissue material has been described and investigated in two previous studies to determine the HA content<sup>27</sup> and RHAMM expression<sup>26</sup> of the lungs; however, only 93 of the 117 samples (from HA study) and 93 of the 94 samples (from RHAMM study) were available for the present work (DNR 53/94 Ethical Review Authority, Uppsala, Sweden). RHAMM expression was determined previously by immunohistochemistry.<sup>26</sup> Lung tissue samples from each of the five lung lobes (performed within 48 h of death) were obtained with parental consent from newborn infants, born between 1990 and 1996 and at a PNA of 0–228 days. The samples were stored in a 4% formaldehyde solution buffered by 10 g/l cetylpyridinium chloride to pH 7.3 until paraffin embedding. Paraffin embedding was done in a vacuum infiltration processor and included dehydration of the samples with a graded alcohol series (70–99.5%) and clearing with xylene (100%). Embedded samples were sectioned (4 µm) by a cryostat and mounted on slides.

### Patient data

Patient data were extracted from archived medical records. Of the 93 infants, 34 were females (37%) and 59 males (63%); 75 infants were born preterm (80%) and 18 at term (20%). All pregnancies were evaluated by ultrasound examination at gestational weeks' 14–17. Four infants died just before birth. The live born children were treated at the neonatal intensive care unit of the Uppsala University Children's Hospital, Uppsala, Sweden. The patient

**Table 1.** Patients' clinical characteristics (n = 93).

	Gestational age (weeks)	Birth weight (g)	Postnatal age at death
Minimum	22	456	7 h
Maximum	41	4720	228 days
Mean	29	1633	14 days
Median	27	1045	2 days
Gestational age (weeks)	n = 93		
21–24	27		
25–28	26		
27–32	12		
33–36	11		
37–40	15		
41–42	2		
Birth weight (g)	n = 93		
456–1000	47		
1001–1500	7		
1501–2000	8		
2001–3000	18		
3001–4000	10		
4001–4720	3		
Postnatal age at death (days)	n = 93		
Unknown	12		
0–1	37		
2–3	12		
4–7	13		
8–14	3		
15–30	9		
>30	7		

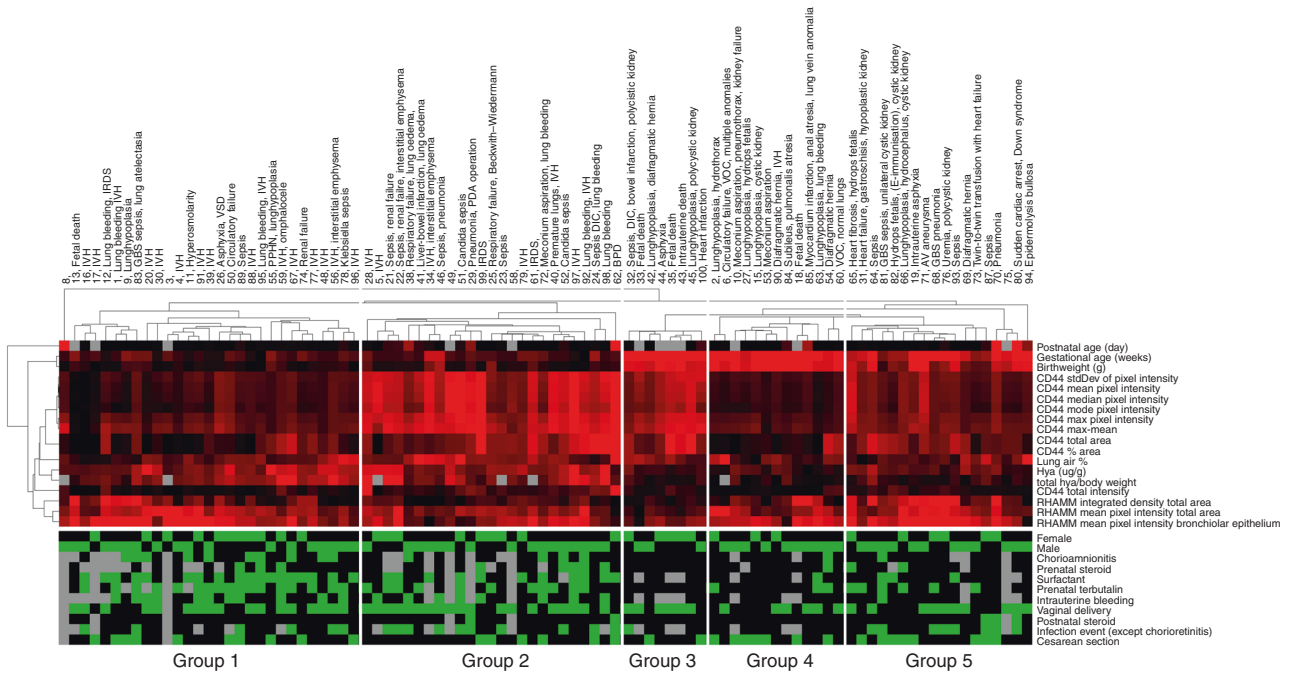
characteristics are represented in Table 1. The causes of death are listed in Fig. 1 and summarized in Table 4.

### HA concentration, RHAMM expression, and air content

HA concentration per body weight, RHAMM expression, and the air content of the lungs were estimated in our previous studies on the same patient samples.<sup>26,27</sup>

### Immunofluorescence staining

Paraffin-embedded sections, after de-paraffinization and rehydration steps, were placed in high-temperature microwave antigen retrieval with citric acid solution (pH 6.0) for 10 min. Sections were then blocked for 30 min with 10% goat serum (Sigma Aldrich®). Tissue sections were incubated 60 min at room temperature with the primary antibody, a monoclonal mouse anti-human CD44 antibody (1:50, clone A3D8, c-7923, Sigma Aldrich®), which recognizes both CD44 isoforms. After washing, sections were further incubated for 60 min at room temperature with the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100, c-349031, Becton Dickinson®). Finally, the cover glass was mounted with Fluoromount G (Invitrogen®). Control sections were blocked with goat serum and stained with the FITC-labeled secondary antibody alone. Representative sections were examined by standard fluorescence microscopy. The same magnification (×60 objective) and exposure time were used for capturing all the images. Three images per section/patient were captured (image type: 8-bit grayscale, image size: 4 megapixels, 2399 × 1599 pixels). Eight-bit images allowed the



**Fig. 1 Patient groups after two-dimensional hierarchical clustering.** Higher intensity in the red color corresponds to a higher value of a certain parameter. Parameters labeled with red pixels were weighted equally in the clustering process. The lower part of the figure (with green pixels) represents clinical data or early clinical interventions that were not included in the clustering analysis. Green pixel = yes, black pixel = no, gray pixels = no data available. Patients were sorted into five groups according to the parameters labeled with red; each group shows an individual pattern. Cause of death is listed above the image.

range of 255 intensity levels; however, the proper exposure time for the whole stack resulted in no saturated pixels.

The staining for RHAMM and CD44 were performed on separate tissue sections at different time points and by different methods, which prevented the analysis of co-expression of the two receptors in tissue regions or on cell types. The immunofluorescence labeling of CD44 did not allow qualitative histological evaluations.

**Software and image analysis**

A total of 279 images were sorted into corresponding stacks and saved in TIFF format. ImageJ<sup>28</sup> was used for semiautomatic image analysis. Working with image stacks during the evaluation process allowed for the comparison between images in the analysis. Higher pixel intensity corresponded to higher CD44 expression. Before analysis, the same threshold window was set on all images in order to filter out any image with pixel values that were too low, usually corresponding to autofluorescence.

**CD44 expression**

Three representative images per section were selected and used for the measurement of CD44 expression. After the relevant threshold value was set for the whole project, the analysis was performed by using the Analyze Particles tool (ImageJ). We chose nine parameters to describe the characteristics of the digital images:

- CD44 mean pixel intensity: mean value of pixel intensity level within the threshold area. Allows for the comparison of CD44 expression levels between patients even if the area with CD44 expression or the tissue-covered area of the section is different;
- CD44 stdDev of intensity: standard deviation of the pixel intensity values used to generate the mean intensity values. Describes the heterogeneity of pixel intensity levels, namely, a higher value occurs if CD44 expression is more heterogeneous within the threshold area;

Clinical parameters	Postnatal age Gestational age at birth Birth weight
CD44 expression	CD44 stdDev of pixel intensity CD44 mean pixel intensity CD44 median pixel intensity CD44 mode pixel intensity CD44 maximum pixel intensity CD44 max–mean CD44 total area CD44 % area CD44 total intensity (integrated density)
Lung air content	Lung air content %
Hyaluronan content	HA µg/1 g lung Total HA/body weight
RHAMM expression	RHAMM integrated density RHAMM mean pixel intensity of total area RHAMM mean pixel intensity of bronchiolar epithelium
Each parameter was weighted equally in the clustering process.	

- CD44 mode pixel intensity: most frequently occurring pixel intensity value within the threshold area, corresponds to the highest peak in the histogram;
- CD44 max pixel intensity: maximal intensity value within the threshold area;
- CD44 median pixel intensity: median intensity value within the threshold area;

- CD44 max–mean: calculated as the difference between the maximal and mean intensity values. Describes the height of intensity peaks within the threshold area in case of high heterogeneity in the threshold area;
- CD44 total area: number of pixels in the threshold covered area;
- CD44 % area: calculated from the threshold area and the whole image area; and
- CD44 total intensity: total pixel intensity (integrated density) in the threshold covered area.

All the nine parameters were estimated for each individual image. The mean values of the corresponding images were calculated in Microsoft Excel.

**Cluster analysis**

The clinical data (GA, birth weight [BW], PNA) were analyzed together with CD44 and RHAMM expression, as well as lung air and HA content by two-dimensional hierarchical clustering (Cluster 3.0 freeware<sup>29</sup>) as previously described.<sup>26</sup> The clustering sorted patients into groups depending on the relationship between their parameters. Eighteen parameters (shown in Table 2) were used and weighted equally in the clustering algorithm. The results were visualized by Java Treeview,<sup>30,31</sup> as a map of color

pixels. Higher intensity in red corresponds to higher value of a certain parameter.

**Statistical analysis**

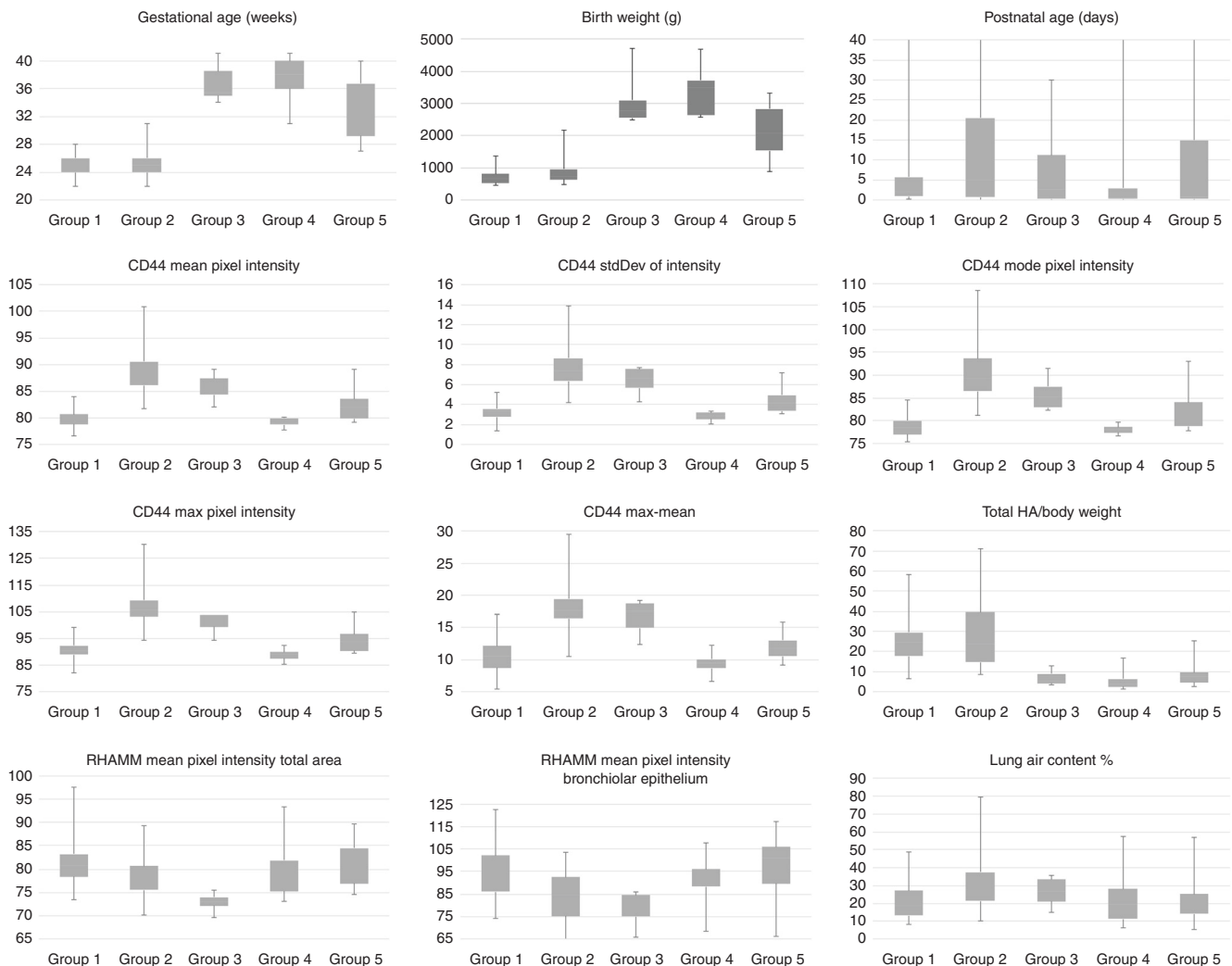
Analysis of variance together with two-tailed *t* test was performed to assess significant differences. Pearson’s correlation was used to show if, and how strongly, pairs of variables were related. *p* < 0.05 was considered as a significant difference.

**RESULTS**

**Clustering of patients**

Ninety-three patients were sorted into five groups by the hierarchical clustering analysis (Fig. 1). Each group was unique and showed an individual visual pattern of red pixels, thereby confirming the utility of the clustering method. The patterns of the individual parameters are shown in Fig. 2.

Term and preterm neonates were sorted automatically into the following groups by the cluster analysis method: Group 1, GA: 22–28 weeks; Group 2, GA: 22–31 weeks; Group 3, GA: 34–41 weeks; Group 4, GA: 31–41 weeks; Group 5, GA: 27–40 weeks; (Fig. 1). All CD44 parameters showed the same pattern among the groups (Fig. 2). All the extremely premature infants showed high HA concentrations, where lung development stages were canalicular or early saccular at birth (Groups 1 and 2,



**Fig. 2 Characteristics of patient groups.** Patterns of individual parameters in different patient groups after hierarchical clustering, visualized by box plot diagrams.

**Table 3.** Additional multiple comparisons of all groups to test for differences detected by the cluster analysis.

	Gestational age	Birth weight	Postnatal age at death	Lung air content	CD44 mean pixel intensity	RHAMM mean pixel intensity	Total HA/body weight
Group 1 vs 2	<b>0.339</b>	<b>0.0613</b>	<i>0.049</i>	<i>0.006</i>	*	<b>0.062</b>	<b>0.269</b>
Group 1 vs 3	*	*	<b>0.508</b>	<b>0.175</b>	<i>0.0001</i>	*	*
Group 1 vs 4	*	*	<b>0.346</b>	<b>0.745</b>	<b>0.141</b>	<b>0.478</b>	*
Group 1 vs 5	*	*	<b>0.110</b>	<b>0.851</b>	<i>0.002</i>	<b>0.926</b>	*
Group 2 vs 3	*	*	<b>0.308</b>	<b>0.184</b>	<i>0.027</i>	*	*
Group 2 vs 4	*	*	<b>0.443</b>	<b>0.070</b>	*	<b>0.576</b>	*
Group 2 vs 5	*	*	<b>0.565</b>	<b>0.023</b>	*	<b>0.111</b>	*
Group 3 vs 4	<b>0.710</b>	<b>0.301</b>	<b>0.814</b>	<b>0.472</b>	*	<i>0.0014</i>	<b>0.650</b>
Group 3 vs 5	<i>0.018</i>	<i>0.016</i>	<b>0.229</b>	<b>0.306</b>	<i>0.006</i>	*	<b>0.444</b>
Group 4 vs 5	<i>0.0058</i>	*	<b>0.294</b>	<b>0.875</b>	<i>0.0002</i>	<b>0.539</b>	<b>0.249</b>
Group 1+2 vs 3+4	*	*	<b>0.195</b>	<b>0.486</b>	<i>0.046</i>	<b>0.053</b>	*
Group 1+2 vs 5	*	*	<b>0.262</b>	<b>0.216</b>	<b>0.121</b>	<b>0.457</b>	*
Group 3+4 vs 5	<i>0.0171</i>	<i>0.00040</i>	<b>0.594</b>	<b>0.317</b>	<b>0.869</b>	<i>0.017</i>	<b>0.348</b>

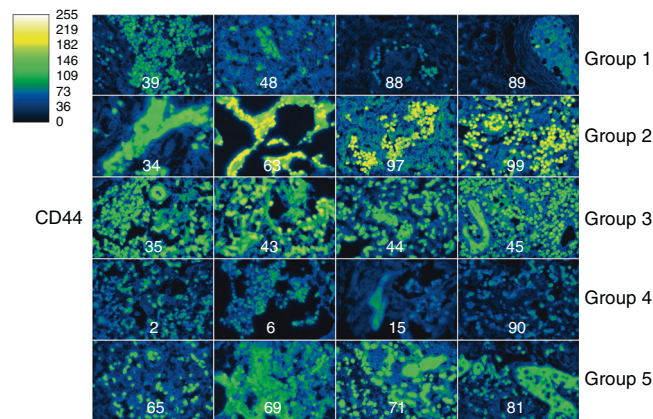
ANOVA was used together with two-tailed *t* test. *p* Values >0.05 indicate that two groups are not statistically different from each other in the specific parameter (in bold); the results confirm the similarities (in bold) and differences (in italics) between Groups 1 and 5 as presented in Fig. 2.

Note: Gestational age and birth weight: Group 1 is similar to Group 2 (extremely preterm at birth) and Group 3 is similar to Group 4 (moderately preterm and term). Group 5 appears alone (mixed population); Postnatal age at death: Group 1 had a shorter postnatal age than Group 2; otherwise no difference was detected between the groups; CD44 expression: Group 1 is similar to Group 4; all the other groups differ from each other; RHAMM expression: all groups are similar to each other except Group 3, which appears as an outlier. (Group 3 has the lowest RHAMM expression, see in Figs. 1 and 2); Lung air content: only a difference between Group 1 and 2 could be detected, similar to the difference in postnatal age at death; Hyaluronan (HA)/body weight content: Group 1 is similar to Group 2 (high HA content) and Groups 3–5 are similar to each other (low HA content).

\**p* < 0.0001.

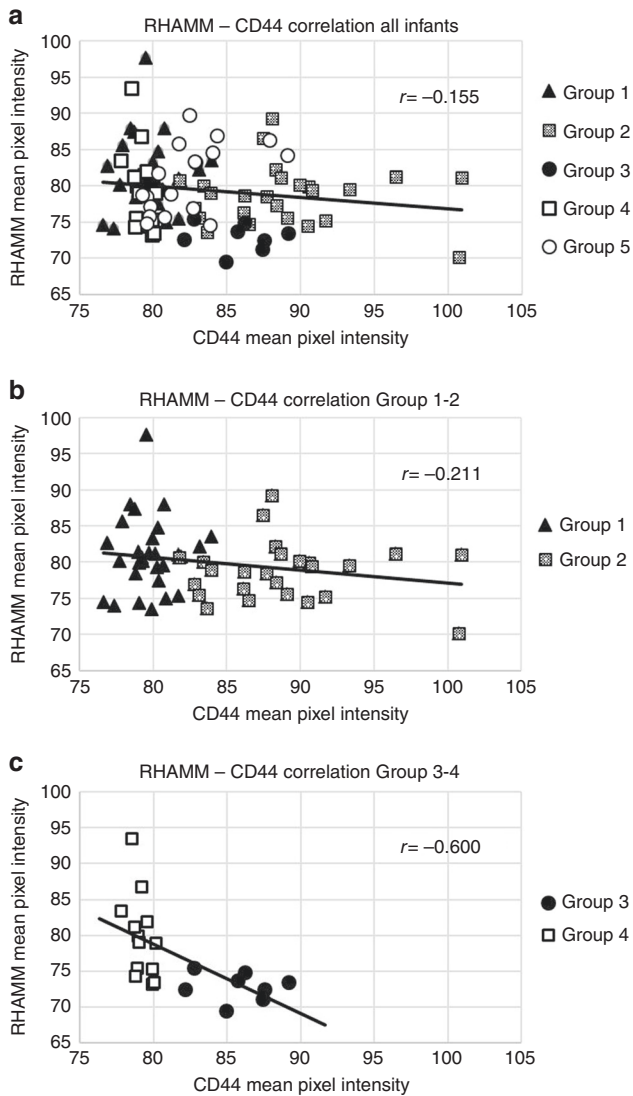
Fig. 2; Table 3). There were no significant differences in RHAMM expression (see RHAMM mean pixel intensity total area) between Groups 1 and 2 (Fig. 2; Table 3). Groups 3–5 represent lungs in the late saccular and alveolar developmental stages at birth and were associated with low HA content (Fig. 2). Group 3 showed the most homogeneous and lowest RHAMM expression and was associated with elevated CD44 levels; however, Group 4 had low CD44 expression and elevated RHAMM levels (Fig. 2; Table 3). RHAMM expression showed heterogeneity within the groups except for Group 3, which had homogeneously low RHAMM expression (Fig. 3). There were no differences in the lung air content and PNA between the groups, except for Groups 1 and 2, where the latter had significantly higher PNA and lung air content (Fig. 2; Table 3). No association between CD44 expression and HA levels were observed, because in cases of high HA levels, both high and low CD44 expression occurred (Groups 1 and 2; Fig. 3). Similarly, lung samples with low HA levels showed both high and low CD44 expression (Groups 3 and 4; Fig. 3). Group 5 was the most heterogeneous group including both term and extremely premature neonates at birth (Fig. 3). Representative lung sections from Groups 1 to 5 are presented in Fig. 3 together with a summary of the levels and heterogeneity of CD44, HA, and RHAMM in each group.

Differences and similarities between the groups  
The statistically significant differences or similarities between the groups are summarized in Table 3. Group 1 showed significant similarities to Group 2 (extremely to very preterm (EVP)), Group 3 was similar to Group 4 (moderately preterm to term (MPT)) in terms of GA, BW, and HA content of the lung. However, Group 5 appeared to be different according to GA and BW. Group 1 shared similarities to Group 4 in its low CD44 expression. When analyzing RHAMM expression, all groups were similar to each other except Group 3, which had the lowest RHAMM expression (Figs. 1 and 2). HA content was significantly higher in Groups 1–2 (EVP) compared to Groups 3–4 (MPT) and Group 5 (Figs. 1 and 2; Table 3), which



**Fig. 3** CD44 expression of representative lung sections (four different infants per group) from Groups 1 to 5. The calibration bar shows how colors represent intensity values. The blue color corresponds to an unspecific low signal (autofluorescence). Green and yellow color represents the threshold area, which corresponds to CD44 expression. Group 1 (GA: 22–28)—low CD44 expression, high HA, and elevated but heterogeneous RHAMM expression. Group 2 (GA: 22–31)—high CD44 expression, high HA, and elevated but heterogeneous RHAMM expression. Group 3 (GA: 34–41)—high CD44 expression, low HA, and lowest RHAMM expression (GA 34–41 weeks). Group 4 (GA: 31–41)—lowest CD44 expression, low HA, and elevated RHAMM expression. Group 5 (GA 27–40 weeks)—the most heterogeneous group including both term and extremely premature neonates at birth; both elevated and low CD44 levels occur, HA is low, and RHAMM expression is moderately elevated but heterogeneous.

corresponds to previous results.<sup>27</sup> No differences could be seen in the pattern of early clinical interventions or conditions between Groups 1 and 2 or between Groups 3 and 4, respectively (Fig. 1, the part labeled with green pixels).



**Fig. 4** Linear correlations between CD44 and RHAMM expression. **a** Whole study group, Groups 1–5 (NS); **b** Group 1 analyzed together with Group 2 (NS); **c** Group 3 analyzed together with Group 4 ( $p = 0.004$ ).

Correlations between CD44 and RHAMM expression  
Statistical similarities in GA, BW, and HA content allowed for cross-over analysis of Groups 1–2 (EVP) and Groups 3–4 (MPT), related to CD44 and RHAMM expression. A strong negative correlation was noted between RHAMM and CD44 expression for MPT ( $r = 0.600$ ;  $p = 0.004$ ; Fig. 4). No such correlations were seen for EVP ( $r = 0.211$ ; NS) or the whole study population ( $r = 0.155$ ; NS). The negative correlation between RHAMM and CD44 appeared to be related to a certain maturation level at birth, as infants in Groups 3 and 4 (GA 31–41) represented lungs in the late sacral or alveolar developmental stages at birth. On the other hand, the negative correlation was specific for Groups 3 and 4 since the correlation disappeared when patients from the other groups with GA 31–41 were included (data not shown). No correlation could be observed between CD44 or RHAMM and GA, PNA, or postmenstrual age when the entire population was analyzed together (Fig. 5). The phenomenon of the negative correlation between RHAMM and CD44 did not seem to be dependent on PNA and lung air content since Groups 1–2 together did not differ from Groups 3–4 in these parameters (Table 3). On the other hand, our results cannot exclude that this interaction between CD44 and RHAMM

expression depends on other postnatal factors and not only on the lung maturity level at birth.

#### Cause of death

All groups were heterogeneous as to their diagnosis at the time of death (Fig. 1, Table 4). Statistical analysis was not feasible as there were too few patients for each diagnosis in each group, but two observations were made. Seventeen patients died owing to serious infections (20.4%); but interestingly, none of these patients were in Group 4 and only 1% was in Group 3 (Table 4). Intraventricular hemorrhage was the most common cause of death in the extremely preterm groups (Group 1: 15%, Group 2: 7.5% of all patients, see in Table 4).

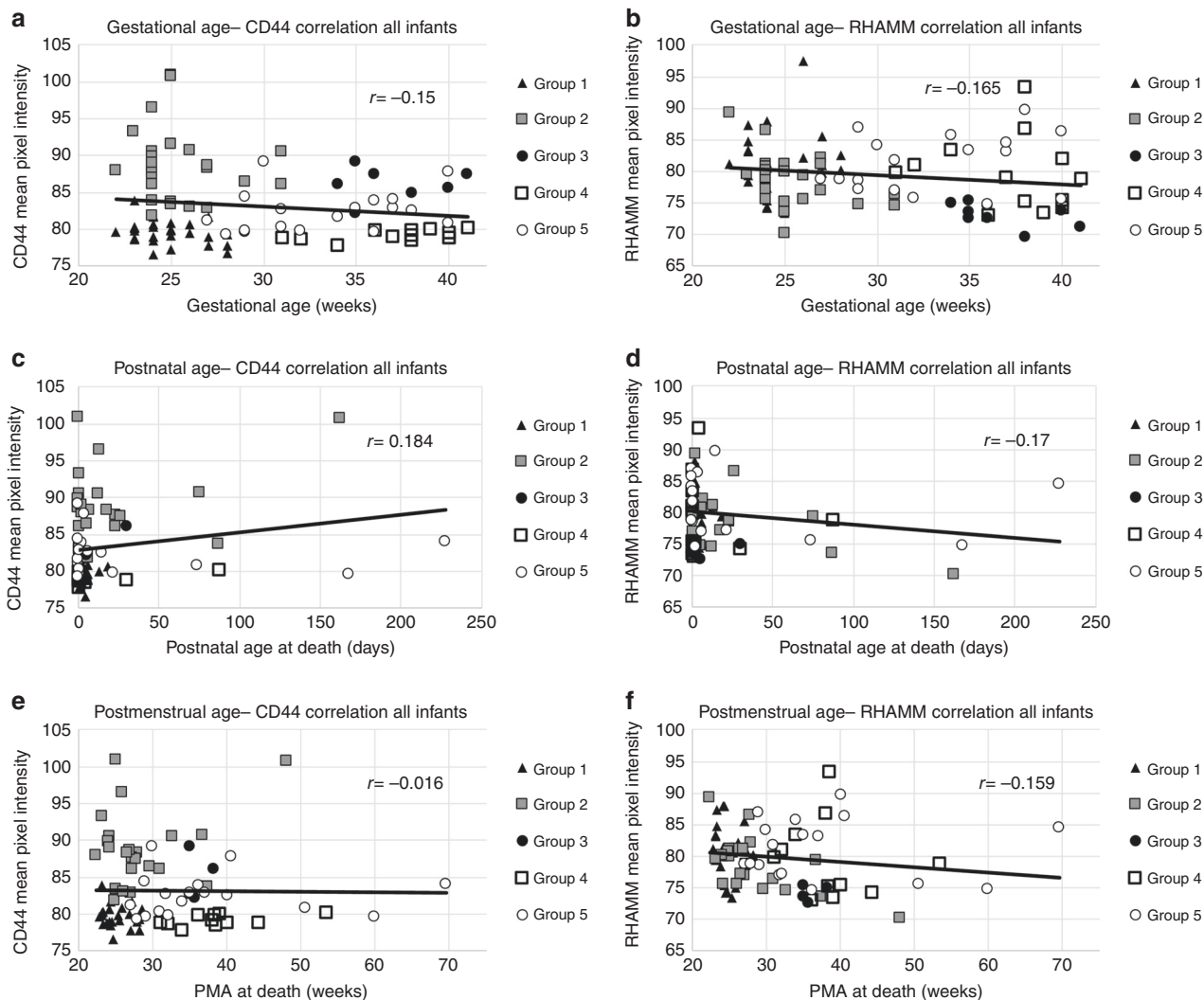
#### DISCUSSION

The components of the ECM constantly interact with cells by serving as ligands for cell receptors that regulate adhesion, migration, differentiation, proliferation, or survival.<sup>32</sup> At least 150 different ECM proteins are expressed in the lung, which participate in a wide range of signaling pathways.<sup>33</sup> Our previous studies suggest that HA as a main component of this matrix and its receptor RHAMM (CD168) may have a pivotal role in lung development.<sup>26,27</sup> The present study improves knowledge on CD44 expression as a possible important actor in lung development together with HA and RHAMM.

Only one study describes the distribution pattern of CD44 in the human lung during development.<sup>4</sup> This study found that CD44 is present in both epithelial and non-epithelial cells during lung development, whereas different CD44v isoforms appear at different developmental stages. Furthermore, they reported reduced expression of the CD44v isoforms in alveolar epithelial and bronchial epithelial cells in fibrotic lung samples. However, this report is not entirely relevant to our study since it contains only morphological descriptions and lacks objective measurements of the expression levels.<sup>4</sup> To our knowledge, our study is the first that measures CD44 expression in the developing human lung in a semiquantitative way and compares CD44 levels between individuals together with RHAMM and HA.

In our previous work, RHAMM expression was determined by immunohistochemistry. For the present study, immunofluorescence was used, since it is a more sensitive tool to measure protein expression levels in cells and the staining is not a result of an amplified process like in immunohistochemistry, thus non-specific signals are less likely. On the other hand, this method also has some limitations as the detailed structure of the tissue is not clearly seen and the cell type expressing CD44 cannot readily be identified.

Underhill et al. found a positive correlation between the number of CD44-positive macrophages and the developmental age in mouse embryos.<sup>17</sup> The increase in the number of CD44-positive macrophages was inversely correlated to the lung HA content and could suggest that these macrophages possibly play a significant role in HA removal.<sup>17</sup> This finding fits together with the observation from Nedvetzki et al. who found that the loss of CD44 allowed enhanced accumulation of the HA in the ECM.<sup>34</sup> We have found that HA content decreases in the human lung during development<sup>27</sup>; however, we could not confirm any correlation between HA and general CD44 expression levels at any lung developmental stage. Nevertheless, general CD44 expression might be hard to interpret, since different cell types may express different levels of CD44, and CD44-expressing cells do not always bind HA. Binding of CD44 to HA is cell specific and depends on the activation state of CD44.<sup>35</sup> We could identify patient groups with low or high CD44 expression by clustering. The determining parameters for sorting patients into groups, besides CD44, were RHAMM expression, HA level, BW, and GA at birth. Interestingly, PNA did not appear as a main determinant for clustering, which



**Fig. 5** Correlations between CD44 or RHAMM expression and gestation age, postnatal age, postmenstrual age in all infants. **a** CD44 vs GA; **b** RHAMM vs GA; **c** CD44 vs PNA; **d** RHAMM vs PNA; **e** CD44 vs PMA; **f** RHAMM vs PMA. No correlations in any of the combinations. Patient groups are marked separately.

implies that intrauterine processes might influence postnatal CD44 expression in the lung for a longer period after birth. Since samples originated from deceased neonates, and there are ethical limits to measuring CD44 expression in healthy fetuses as controls, we are lacking data on normal levels of CD44. Still, one animal model describes increasing CD44 levels during embryonal lung development in mice.<sup>17</sup>

Several studies suggest that RHAMM-mediated signaling can compensate for CD44 functions. Pilarski et al. suggests that RHAMM- and CD44-mediated cell adhesion and motility may function reciprocally rather than in an overlapping manner.<sup>36</sup> Nedvetzki et al. described that arthritis in wild-type mice is CD44 dependent, whereas in CD44-knockout mice, it is RHAMM dependent.<sup>34</sup> These findings are not in conflict with our main finding, as we could identify patient groups where a negative linear correlation was observed between RHAMM and total CD44 expression, a phenomenon that appeared in moderately preterm and term neonates but not in extremely preterm neonates. These results may suggest that RHAMM signaling can be upregulated in the presence of low CD44 levels if the lung development has passed the early sacular stage. Another explanation could be that CD44 and RHAMM coexist; however, CD44 functions may be

dominant under certain clinical circumstances and lung developmental stages.

Leng et al. determined the spatial distribution and gene expression of CD44, RHAMM, and other HA-related proteins on embryonic days 10.5–12.5 in murine forelimbs, showing that HA and CD44 expression remained high while RHAMM decreased during development.<sup>37</sup> In the same study, anti-CD44 inhibited the proliferation of connective tissue cells and muscle progenitors, whereas anti-RHAMM had no effect.<sup>37</sup> Several studies indicate that not only RHAMM but other widespread mechanisms can also compensate for the loss of CD44.<sup>38</sup> The loss of CD44 function in the late developmental stages might not be tolerated in the same way as in the early developmental stages.<sup>39</sup> The decrease of CD44 expression early in embryogenesis might result in the induction of genes that can compensate for CD44 in some instances.<sup>38</sup> Thus the compensation for reduced CD44 expression might not be necessarily connected to RHAMM during the early lung developmental stages. Furthermore, RHAMM does not need to have the common trunk with CD44 in cell signaling pathways during early development, and RHAMM can also act as a co-receptor for growth factor receptors.<sup>40</sup> This could explain why RHAMM expression did not match CD44 expression changes in our

**Table 4.** Distribution of causes of death in all the groups.

%	Group 1	Group 2	Group 3	Group 4	Group 5	Total
Asphyxia	1.1		1.1		1.1	3.3
Bowel infarction		1.1				1.1
BPD		2.2				2.2
Circulatory failure	2.2		1.1	4.3	5.3	12.9
Fetal death	1.1		3.2	1.1		5.4
IRDS	1.1	5.3				6.4
IVH	15.0	7.5		2.2	1.1	25.8
Lung hypoplasia	2.2	2.2		6.4	2.2	13.0
Meconium aspiration				2.2		2.2
Pneumonia		2.2			2.2	4.4
Sepsis	3.2	6.4	1.1		5.3	16.0
Renal failure	1.1				1.1	2.2
No data	2.2	2.2			1.1	5.3

Causes of death were determined by the combination of clinical features and autopsy. The numbers represent the proportion of patients in percentage compared to the total number of patients ( $n = 93$ ). No data: autopsy was not performed.

patients during their early lung developmental stages. Since HA content decreases during lung development,<sup>27</sup> the question arises whether HA is a determinant factor for how RHAMM can compensate CD44 signaling in the developing lung.

The correct interpretation of our results needs to take into account some limitations. The number of patients in the separate groups were not enough to uncover possible significant differences in clinical parameters. CD44 is expressed in a wide variety of cells, including circulating white blood cells,<sup>41</sup> which could lead to rapid changes in the total CD44 expression of the lung. Thus some factors other than lung development might be related to the changes in CD44.

## CONCLUSIONS

Several previous studies indicate a possible interaction between CD44 expression and the expression of RHAMM; our study also suggests a close correlation between CD44 and RHAMM expression beginning already during the late sacular stage of lung development at birth. Although CD44 and RHAMM are expressed in the more immature lungs of extremely and very preterm infants, this expression seems to be influenced by postnatal factors, possibly related to evolving lung disease or the postnatal maturation of receptor expression in different lung tissues.

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## AUTHOR CONTRIBUTIONS

L.M. participated in the design of the study; was responsible for digital image and clustering analysis, statistical analysis, and interpretation of data; and preparing the first draft of the manuscript. R.C.S. participated in the design of the

study, the interpretation of data, and revision of the manuscript. A.J. participated in the initial phase of the study (HA), the interpretation of data, and revision of the manuscript. R.S. conceived the study, participated in its design, was responsible for the immunostaining and the collection of clinical data, and participated in the interpretation of data and the preparation/revision of the manuscript.

## ADDITIONAL INFORMATION

**Competing interests:** The authors declare no competing interests.

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