

Presence of N-acetylneuraminic acid in the lung during postnatal development

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ABSTRACT

Sialic acids, particularly N-acetylneuraminic acid (Neu5Ac), are present as terminal components of rich and complex oligosaccharide chains, which are termed glycans, and are exhibited on the cell surfaces, especially on epithelial cells. Crucial in the ‘social behavior’ of the cell, sialic acids play vital roles in many physiological and pathological phenomena. The aim of the present study was to separate, identify, and quantify Neu5Ac in purified lung membranes from 4-, 14-, and 21-day-old animals, followed by the statistical analysis of these results with our previously reported data (0-day-old and adult results). Complementary, ultrastructural methodologies were used. The differences in the Neu5Ac values obtained across the examined postnatal-lung development relevant ages studied were found to be statistically significant. A substantial increase in the mean level of this compound was found during the period of ‘bulk’ alveolarization, which takes place from postnatal day 4 to 14 (P4-P14). The comparison of the mean levels of Neu5Ac, during microvascular maturation (mainly between P12 and P21), reveals that the difference, although statistically significant, is the least significant difference among all the pair-wise differences between the developmental stages. The presence of sub-terminal N-acetylgalactosamine (GalNAc)/Galactose (Gal) residues with terminal sialic acids on the bronchioloalveolar cell surfaces was confirmed using lung ultra-thin sections of adult and 0-day-old animals. These results showed that, although Neu5Ac levels increase throughout postnatal lung development, this sialic acid was substantially added to epithelial cell surfaces during the “bulk” alveolarization period, while its presence was less important during the microvascular maturation period. Bearing in mind that sialic acids are negatively charged and create charge repulsions between adjacent cells, we hypothesized that they can substantially contribute to postnatal alveolar formation and maturation.

Key words: Sialic acids; N-acetylneuraminic acid; lung development.

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Contributions: MFM, involved in TEM and HPLC methodologies, in the state of the art, in the results discussion and in text elaboration; MSR, involved in the statistical analysis and visualization of results; AHF, involved in HPLC methodologies and in the results discussion; CAG, involved in discussion and writing the paper. All the authors have read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: None.

Ethics approval and consent to participate: All the procedures were in accordance with the ethical recommendations for the use and handling of laboratory animals, as established by the Faculty of Medicine in Coimbra, and according to the ‘General Ethical Principles on Experiments with Animals’ that was conferred by the National Portuguese Veterinary Board (Ordinance n. 1005/92).

Introduction

The lung of air-breathing animals undergoes dramatic changes during development. From a solid organ and a glandular-like organ, it becomes an aerial structure. In mammals, the generation of terminal alveoli occurs mostly during the postnatal period.¹⁻⁵ In humans, the alveoli development begins in the final phase of gestation. In rats, it begins later, yet after birth. However, during alveolarization, rat lung undergoes similar modifications as those of humans, making rats one of the most useful animal models in this field.⁶

Alveolarization comprises a biphasic behavior with a first phase termed classical or “bulk” alveolarization, in which alveoli are formed apace, and a second phase, named continued alveolarization, which involves a slower formation of alveoli. These phases overlap and, in rats, the first phase elapses from postnatal day 4 to day 14 (P4-P14). The second phase of alveolar formation, (continued alveolarization), occurs from P14 until adulthood. Meanwhile, the microvascular maturation occurs.⁷ According to Burri,⁸ it is very difficult to assess the beginning and ending of this process. The microvascular development is the result of the sum of multiple focal fusions between the two capillary layers. In rats, it occurs mostly during the end of the second week and during the third week of postnatal development (between P12 and P21).⁸ During the process of alveolarization, dramatic structural and architectural lung transformations occur to provide optimal diffusion between air and blood. To perform respiratory and non-respiratory functions, mammalian lungs present a large epithelial surface area, especially lining the airway and vascular compartments. Airway epithelial cells are diverse and like all the eukaryotic cells, have an intricate surface coat of complex carbohydrates, which are termed glycans and which are directly or indirectly linked to cell fate and differentiation.⁹⁻¹⁵ One of the classical terminal modifications of glycans is sialylation. As such, sialic acids are found on N-glycans, O-glycans, and glycolipids, and also occur on O-fucose and O-mannose glycans that are present in the extracellular matrix, cell cytosol, and the outer leaflet of plasma membranes.¹⁶ Sialic acids are highly negatively charged residues, and N-acetylneuraminic acid (Neu5Ac) is the most common sialic acid in humans. Due to their terminal position in the glycan chains present on the cell surfaces, sialic acids are the first molecules to encounter other molecules being crucial in the “social behavior” of the cell. They also contribute to a meaningful net negative charge on the cell surface and are implicated in important biophysical effects.¹⁷⁻²¹

During the last decades, several approaches have been developed to study cell surface glycans. Due to their complexity, this issue should be studied using more than one method.²²⁻²⁵ Although lung development and many of the mechanisms involved in the process have been investigated during the years, studies on the presence and possible contribution of glycans and particularly of sialic acids, in this process are sparse.^{18,19,26,27} Thus, aiming for a better understanding of the lung glycocalyx composition during postnatal lung development, we separated, identified, and quantified the sialic acid, Neu5Ac, in purified lung membranes from 4-, 14-, and 21-day-old rats using high-performance liquid chromatography (HPLC) methodologies. The results obtained were statistically analysed and also compared to data from Martins *et al.* (personal observation and manuscript in preparation) and

previous reported data that were obtained in 0-day-old and adult animals.²⁸ Complementarily, in order to infer the presence of terminal sialic acids, we studied the labeling pattern of the lectin *Glycine max* (common name soybean agglutinin, SBA), with specificity to N-acetylgalactosamine (GalNAc)/galactose (Gal) residues, in neuraminidase non-pre-treated and pre-treated ultra-thin lung sections.

Materials and Methods

Animals and tissue collection

Animals older than 8 days and pregnant female rats were supplied by the Charles River Laboratory Animals in Barcelona, Spain.

Animals were anaesthetized with intraperitoneal sodium pentobarbital (25 mg/Kg) and lungs were collected at 0-, 4-, 14-, and 21- days after birth, as well as for adult animals. The anaesthetized animals were submitted for 90 s to lung perfusion *via* the right ventricle with 0.1 M phosphate buffered saline solution (PBS) at a pH of 7.4 and a flow rate of 1 mL/min to 5 mL/min, according to the age. All the procedures were in accordance with the ethical recommendations for the use and handling of laboratory animals, as established by the Faculty of Medicine in Coimbra, and according to the ‘General Ethical Principles on Experiments with Animals’ that was conferred by the National Portuguese Veterinary Board (Ordinance n. 1005/92 from 23th October).

Animals for transmission electron microscopy

A total of two adult and four 0-day-old animals were sacrificed. After the initial perfusion, the fixation process continued with a 90 s (for 0-day-old animals) to a 5 min perfusion (for adult animals) with 4% formaldehyde in PBS at a pH of 7.4 with 0.5% glutaraldehyde at a flow rate of 5 mL/min and 1 mL/min, according to the age. Collapsed lungs were excised and 2 mm³ pieces of the lungs were immersed for 5 h in fixative and then rinsed in TBS. Specimens were embedded in agar and 50 µm sections were cut on a ‘Smith Farquhar’ tissue sectioner (Sorvall Co., USA) and rinsed in TBS for 30 min.

Animals for HPLC

Lungs from 4-, 14- and 21-day-old animals after birth were collected. For each experiment, two to five animals, based on the animal age, were sacrificed per experiment, which results in a total of 9 experiments (three experiments per age) and 30 sacrificed animals. After the initial perfusion with PBS, the process was completed with a 1 to 2-min perfusion process at the same flow rate with the following homogenization buffer: 10 mM of PBS at a pH of 7.4 with 30 mM NaCl, 1 mM MgCl₂, 0.005 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 0.02% of sodium azide (NaN₃), and 5 µg of DNase (D4527; Sigma-Aldrich, Saint Louis, MO, USA). Collapsed lungs were excised, immersed, and rinsed in the homogenization buffer. Lung fragments were dissected, and zones of great vessels and main bronchi were discarded. Samples from different pulmonary lobes were then processed for HPLC (see below).

Table 1. Lectin characteristics.

Species	Common name	Abbreviation acronym	Concentration used	Saccharide affinity	Inhibitory saccharide
<i>Glycine max</i>	Soybean	SBA	20 µg/mL	GalNAc>Gal	GalNAc

Ultrastructure lectin histochemistry

After being fixed, the lung pieces of approximately 1 mm³ were embedded in agar, and 50 µm sections were subsequently incubated for 16 h at +4°C with the horseradish-peroxidase-conjugated lectin that was diluted to the appropriate concentration (Table 1), as previously described.²⁹ After the incubation period, tissue sections were developed in 10 mL of TBS containing 12.5 mg of DAB and 7.5 µL of H₂O₂ at 30% for 10 min and post-fixed in 0.1 M cacodylate buffered at a pH of 7.4 with 1% osmium tetroxide for 1 h. Then, tissue sections were dehydrated in a graded ethanol series and embedded in an epoxy resin. Ultrathin sections of 50-70 nm thickness, were cut and collected on copper grids. To not increase the labeling pattern, the biological material was observed without uranium-acetate or lead-citrate contrasts, as these salts would have masked the cytochemical reaction.

Control for lectin staining

The reaction specificity for transmission electron microscopy (TEM) studies was evaluated by pre-incubating the horseradish peroxidase-conjugated SBA with the corresponding inhibitory sugar (0.4 mM; Table 1) for 50 min prior to staining.

Sialidase digestion

In certain experiments, sialic acid residues were removed using neuraminidase digestion, prior to use of the lectin. Thus, thick sections that were to be further processed for TEM were incubated for 18 h at 37°C in a 0.1 M acetate buffer solution (pH 5.5), containing 0.15 M NaCl, 40 mM CaCl₂, and 1 U/mL of neuraminidase Type X from *Clostridium perfringens* (Sigma-Aldrich).

Plasma membranes

Preparation of plasma membranes

Lung samples for HPLC studies were finely minced, maintained in a 3-fold excess of homogenization buffer and processed according to Maeda *et al.* and adapted to our biological material, as previously described by our research team.^{28,30} After the final centrifugation, the different pellets were stored at 4°C and processed for HPLC for a period that did not exceed 16 h.

Control of plasma membranes

The pellets were immersion-fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 90 min, and small pieces (1-2 mm³) were post-fixed in 1% osmium tetroxide in 0.1 M PBS (pH 7.4) for the same time, and processed as previously described with the same specifications.²⁸ Ultrathin sections were routinely counterstained with uranyl acetate/lead citrate.

HPLC studies

Equipment, reagents, and solutions

Sialic acid hydrolysis, derivatization, and HPLC separations

Purified membranes from 4-, 14-, and 21-day-old animals were used and processed according to studies of Anumula,³¹ and were adapted and optimized to our biological samples. All details regarding this adaptation - as well as the particulars in terms of the reagents, solutions, material, and HPLC system that were used in the present investigation - were already thoroughly described in previous papers.^{28,31}

The detector was adjusted for reading at 214 nm, and quantification was performed by external calibration. Standard

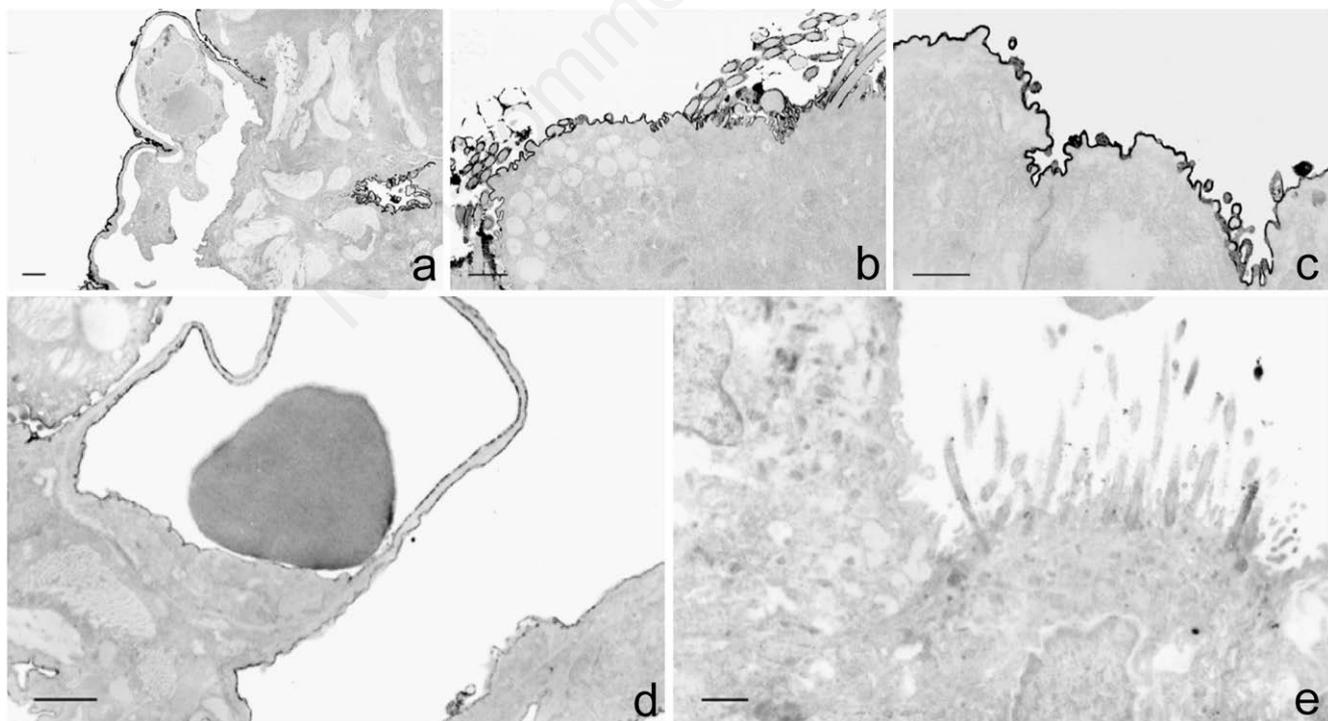


Figure 1. Pneumocytes (a) and ciliated and non-ciliated cells (b) of adult rat show a strong affinity to SBA when lung sections were pre-treated with neuraminidase. A positive reaction on the surfaces of 0-day-old airway epithelial cells is also evident (c). The surface of both pneumocytes and endothelial cells of adult animals show a weak reaction in non-pre-treated sections (d). No reaction is observed in a control section after digestion with neuraminidase (e). Scale bar: 1 µm.

curves were performed using 2 mL of Neu5Ac and increased to 10 mL with solution A at a final concentration of 4 µg/mL for each standard. Standard solutions with 0.5, 2 and 4 µg/mL of each residue were used and 500 µL of each dilution were injected onto the chromatograph. Three injections were performed for each concentration. As previously described, the mean peak area from a known standard was compared to each sample peak area in order to achieve quantification.³²

Statistical analysis

The mean levels of Neu5Ac were studied in six experimental groups that correspond to the following developments stages: 0-day (n=10, results submitted); 4-days (n=3); 14-days (n=3); 21-days (n=3); and >60 days (n=15, data previously published).

Differences in the mean levels of Neu5Ac across these experimental groups were assessed using a one-way analysis of variance (ANOVA) with fixed effects. The ANOVA hypothesis of homogeneity of variances in the groups and normality of residuals was verified using dedicated visualisation tools and formal statistical hypothesis tests. For testing homogeneity of variances, the following tests were adopted: O'Brien, Brown-Forsythe, Levene and Barlett. In terms of the Gaussianity of the residuals, we applied the Shapiro-Wilk test. The assumption of statistical independence of observations was secured in the manner experiments were planned and conducted. The post hoc analysis of differences between groups was completed using the Tukey-Kramer HSD method for multiple comparisons, in order to control for the overall significance level of the analysis, which was established at 0.05 (5%). This test protects against the inflated levels of Type I errors given all the combinations of pairwise comparisons. It is thus a conservative analysis, which ensures that all differences detected as statistically significant are indeed relevant. Statistical analysis was performed using the JMP® Pro software, from the SAS (version 14.0.0 64-bit) software package.

Results

Ultrastructure lectin histochemistry

As uranyl-acetate or lead-citrate contrast could mask the cytochemical reaction, they were not used in the ultrastructural studies. Thus, the positive staining observed on the cell surface was only due to the lectin-labelled/DAB/osmium reaction.

The cytochemical reaction observed after pre-treating adult rat lung sections with neuraminidase before the incubation with horseradish peroxidase-conjugated lectin revealed an evident layer of staining on the free surfaces of type I and type II pneumocytes in adult animals (Figure 1a) as well as on the surfaces of ciliated and non-ciliated cells (Figure 1b). The same labelling pattern was present in 0-day-old animals. Thus, we observe an evident positive reaction on the surfaces of 0-day-old airway epithelial cells after sections had been pre-treated with neuraminidase prior to lectin incubation (Figure 1c). However, when adult lung sections were

not pre-treated with neuraminidase, the reaction was weak and present on the surfaces of both pneumocytes and endothelial cells (Figure 1d). Thus, this labelling pattern was not only evident in adult rat ultrathin lung sections, but also in 0-day-old animals, showing the presence of sub-terminal GalNAc>Gal residues with terminal sialic acids already after birth.

A complete absence of staining was observed when adult rat lung sections were pre-incubated with SBA and the corresponding inhibitory sugar (Figure 1e, control).

Control of plasma membranes

The TEM methodology carried out and the ultrastructure images revealed a high concentration of membranes in the samples that were used for HPLC studies (Figure 2).

HPLC studies

As previously reported, after optimizing the method, Neu5Ac as O-phenylenediamine derivatives, which were obtained from purified membrane fractions, were eluted at 23-24 min.²⁸ The mean values for Neu5Ac obtained in the three different age-moments are exhibited in Table 2. Formerly, our team²⁸ reported that the mean value of Neu5Ac in adult and 0-day-old rat lung purified membranes was 12.26 µg/mg and 5.43 µg/mg of proteins, respectively. These data were included in our present statistical analysis.

Statistical analysis - Comparison of Neu5Ac data across the different ages during postnatal lung development

Differences in the mean values of Neu5Ac that were obtained in purified lung membranes from 0-, 4-, 14-, 21-day old and adult animals, were assessed using a one-way ANOVA with fixed effects. This test signaled the existence of statistically significant differences ($P < 0.0001$).

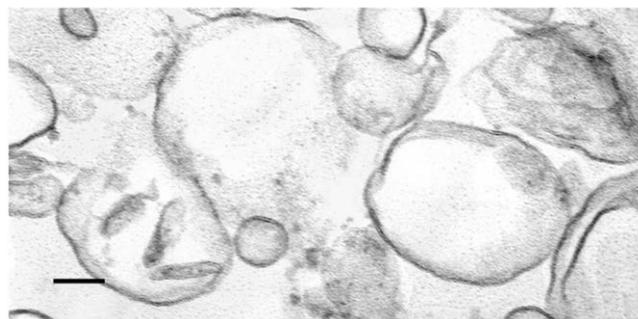


Figure 2. Ultrastructure image of plasma membranes isolated from adult rat lung samples. Scale bar: 1 µm.

Table 2. Quantitative results of Neu5Ac in purified lung membranes from 4-, 14-, and 21-day-old animals, obtained in three separate experiments. The mean values of Neu5Ac are shown.

Age	Experiment results (µg Neu5Ac/mg prot)			Mean (µg Neu5Ac/mg prot)
4-day-old	6.96	6.50	7.33	6.93
14-day-old	9.12	9.81	9.04	9.32
21-day-old	10.68	11.39	9.70	10.59

To analyze which groups indeed differ, a *post-hoc* analysis was conducted, beginning with the visualization of the groups mean levels and respective confidence intervals. This is depicted in Figure 3, in which the diamonds convey information on the mean level (center line) and the confidence interval (top and bottom of the diamond); further, the horizontal size of the diamond is proportional to the sample size. The circles at the center implement the Newman-Keuls method to compare group means, which consist of the following: each experimental group corresponds to a circle; experimental groups with means that are significantly different, do not intersect, or intersect only slightly (there is a quantitative criterion based on the angles for the tangent lines at the interception point, which is not further explored in this paper, as the aim is only to perform a preliminary exploratory inspection); if circles intersect extensively or are nested, then the means of the experimental groups are not significantly different. The Normal quantile plot on the right allows for a visual check of the normality of data in each experimental group: straight lines are expected for Gaussian distributed variables; furthermore, parallel lines indicate equal variances, so this plot is also useful for a preliminary check of the homogeneity of the variances. From the plot on the right it is possible to observe that data for each experimental group present a linear trend with good approximation, and the underlying lines are approximately parallel, which indicates that the Gaussianity and variance homogeneity assumptions are likely to be verified. These aspects were confirmed with formal statistical tests for the Normal distribution (Shapiro-Wilk for the residuals, $P=0.2310$) and homogeneity of variances (O'Brien, $P=0.1139$; Brown-Forsythe, $P=0.1198$; Barlett, $P=0.1222$; Levene, $P=0.0441$). For the homogeneity of variances, all statistical tests, except for the Levene's test, corroborate its validity. This, however, may indicate a possible mild departure from normality. Therefore, we have also applied the Kruskal-Wallis (rank sum) test, which confirmed the existence of (at least one pair of) statistically significant differences in the medians of the groups, as occurs in the initial analysis ($P<0.0001$).

Analyzing Figure 3 (plot on the left side), a non-linear increasing trend in the levels of Neu5Ac along the development stages is evident, which possibly leads to three or four subgroups with statistically significant different levels of this compound (see also Figure 4, because in Figure 3 the groups in the X-axis are not consistently spaced to their actual differences, which distorts the real

evolution trend). More discriminating power could eventually be achieved using larger samples for the intermediate stages (4- to 14-days). Moreover, a step increase of Neu5Ac from 8 to 14 days is evident. To determine which stages do present a difference that is statistically significant (corrected for multiple comparisons), a Tukey-Kramer HSD method for multiple comparisons was performed, where all the paired differences were analyzed. Table 3 presents the details for all the pairwise comparisons and provides information on which experimental groups have means that can be considered statistically different. All differences were found to be statistically significant, and the least significant difference regards the comparison of Neu5Ac levels at 14 vs 21 days.

Discussion

The emphasis of most studies on lung development have been centered on the epithelium, given its vital function in the lung. Alveolar formation involves a systematic coordination of different cell types and important changes take place in the distal epithelium.^{3,5,10,33}

Sialylation has been reported to play important roles in cell fate decisions during development. Further, the contribution of sialic acids during fertilization and early embryogenesis is well-documented.^{13,34,35} In addition, the importance of sialoglycans during organogenesis was described. Different authors reported the influence of sialic acids on nervous system development, brain development, kidney formation, and podocyte maturation.³⁶⁻⁴⁰

The mechanisms that underlie lung development, and the development of other organs, include a variety of multiple mediators, namely growth factors and transcription factors, which are expressed in different phases of the developmental process. It is known that the expression of these mediators is directly or indirectly related to sialic acid content.^{6,34,41}

In human fetuses, a slight decrease in total sialic acid expression was reported to occur in lung epithelial structures during intra-uterine development with a relative minimum before birth. An increased expression of sialic acid residues during development of alveoli and differentiation of pneumocytes I and pneumocytes II - with the latter expressing high amounts of sialic acids - was also described.⁴²

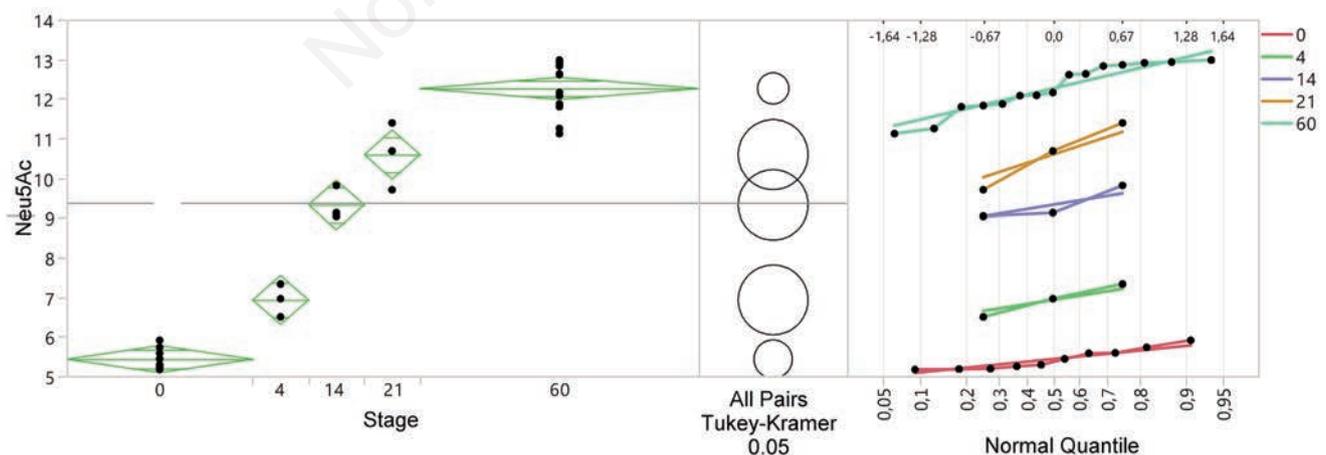


Figure 3. Graphical representation of the Neu5Ac levels for the experimental groups. The diamonds on the left plot represent the means and their 95% confidence intervals. The circles on the center refer to the Newman-Keuls method for comparing group means. The plots on the right are the Normal quantile plots for the data from each experimental group (a linear behavior is expected for variables following a Gaussian distribution).

Martins *et al.* used light microscopy cytochemistry to reveal a strong increase in the intensity of the reaction when 1-, 4-days-old and adult rat lung tissue sections were pre-incubated with neuraminidase prior to SBA staining, suggesting the presence of terminal sialic acids.⁴³ In 2002, Martins *et al.* also demonstrated that the SBA labelling pattern after sialidase treatment at the ultrastructural level was much stronger and predominantly located on the surface of the alveolar epithelial cells in adult rat lung sections.²⁶ The present study confirms the ultrastructural results in adult animals and further evidences the same labelling pattern in 0-day-old lung sections. The evidently higher reaction intensity that is observed when 0-day-old and adult lung sections are treated with neuraminidase before SBA incubation reinforces the presence of sub-terminal N-acetylgalactosamine/galactose residues that are mainly “capped” with terminal sialic acids. Therefore, the capping sialic acids already mask the sub-terminal residues, after birth. As previously shown, this pattern is more evident in the airway compartment than in the endothelium, with the luminal surfaces of pneumocytes showing a stronger affinity for SBA when sections are pre-treated with neuraminidase, thus, suggesting a higher concentration of sialic acids at this level. The stronger SBA staining after neuraminidase treatment of the sections was not only evident on the surface of the alveolar cells but also coating the bronchiolar cells. Thus, our data, at the ultrastructural level, demonstrated and confirmed the presence of terminal sialic acids that “mask” sub-terminal N-acetylgalactosamine/galactose residues on the surface coat of bronchioloalveolar adult and 0-day-old cells. To continue towards a better understanding of the lung epithelial glycocalyx composition, we have also previously quantified Neu5Ac residues in purified lung membranes of adult and 0-day-old rats. We demonstrated a statistically significant increase in Neu5Ac, upon comparing the data from the two age-periods.²⁸

Given the lung developmental phases and the underlying relevant age-periods, the results obtained in lung purified membranes from 4-, 14-, and 21-day-old animals were statistically analysed, and compared to one another and with data that were previously reported in adult and 0-day-old animals. To the best of our knowledge, this work represents the first quantitative investigation and statistical analysis of Neu5Ac obtained in purified lung membranes at relevant age-moments of postnatal development, complemented with ultrastructural data. All subgroups that corresponded to the different developmental stages (0-, 4-, 14-, 21-day-old and adult animals) showed statistically

significant differences in the levels of this compound (Figure 5). We now demonstrate, with more experimental data and stronger statistical evidence, the presence of a significant increasing trend in Neu5Ac during postnatal lung development. These results are in accord with the postnatal lung development stages that have been comprehensively described by several authors.^{7,8,44-47} More specifically, the comparison of 0- and 4-day-old animals – the period in which there is no alveolar formation and the structural studies have shown that the rat lung is in a saccular stage – corresponds in this study to a mild evolution in Neu5Ac. Comparing the Neu5Ac quantifications that were obtained in lung isolated membranes from 4-, and 14-day-old animals - the period in which the alveolarization process is established and the alveoli are formed at high rate^{4,7,48,49} it was verified that there was a significant increase in the Neu5Ac mean level, suggesting that this sialic acid is substantially added to epithelial cell surfaces during this period of lung development. Moreover, a steep increase of Neu5Ac from the 8 to 14 days is evident, which is in accord with our previous reported data.⁴³

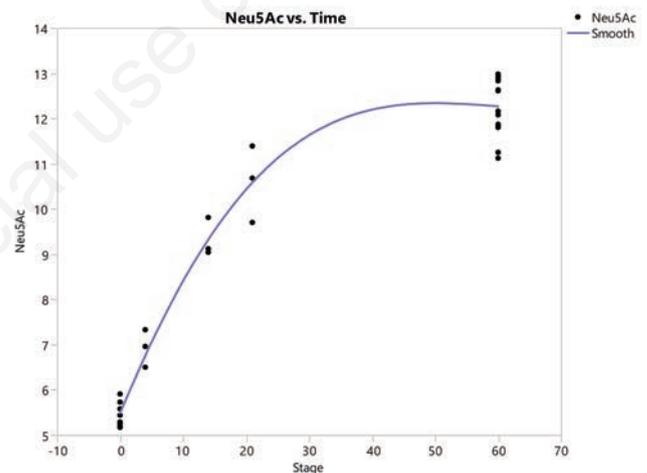


Figure 4. A time series plot of experimental data.

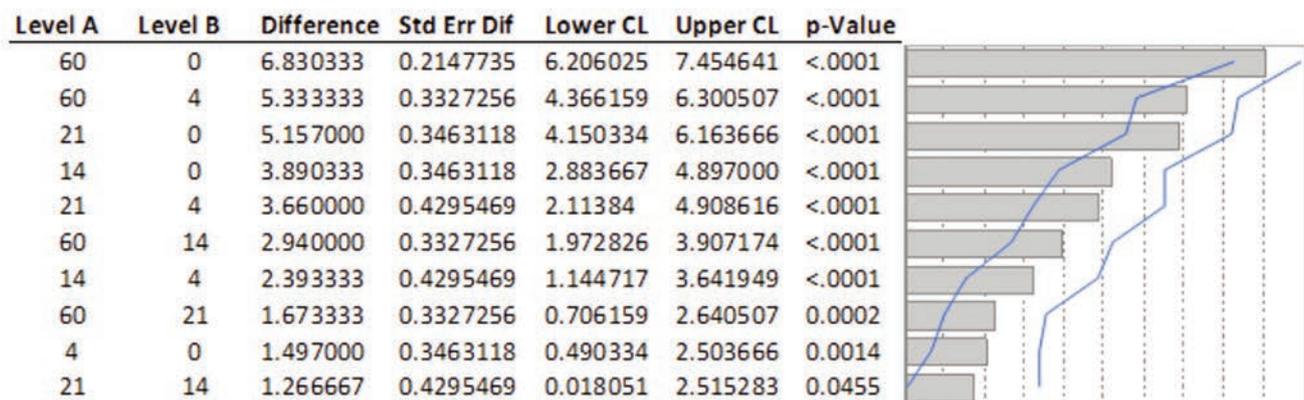


Figure 5. Details for the Tukey-Kramer HSD analysis. Differences between group means are ordered by decreasing magnitude. P-values lower than 0.05 indicate significant differences between the groups indicated in the first two columns (stage).

Bearing in mind that the microvascular maturation occurs in rats mainly during the third week of postnatal lung development, our results show that, when comparing Neu5Ac quantifications from purified lung membranes of 14- and 21-day-old animals, the difference, although statistically significant (P-value = 0.0455), is the least significant one among all pairwise differences between the development stages. This could imply a less important presence of this sialic acid during the microvascular maturation, when the double-layered capillary network fuses to a single-layered one, thus suggesting that, despite its presence on the endothelial cell surfaces, sialic acids may have a much more important presence in the alveolar compartment. As previously reported by different authors, alveoli formation continues after the microvascular maturation is completed.^{8,47,50-52} In our investigation, we sought to elucidate the presence of Neu5Ac during this period by comparing Neu5Ac data from 21-day-old and adult animals. A significant difference was found, which indicates that Neu5Ac may be continuously added onto epithelial cell surfaces during continued alveolarization.

According to Hogan *et al.*¹⁰, after birth, mechanical factors are key regulators of alveolar development.¹⁰ On the other hand, it is known that sialic acids have major biophysical effects providing negative charges and hydrophilicity to the cell surfaces, and are involved in charge repulsion between adjacent cell surface molecules.^{18,53-56} Our quantitative data on the purified lung membranes at relevant age-moments of postnatal lung development, showed a non-linear increasing trend in the levels of Neu5Ac across all the developmentally relevant ages examined. Thus, all subgroups that correspond to the different development moments were found to show statistically significant difference in the levels of this compound. However, the highly significant increase in the Neu5Ac mean level obtained in lung isolated membranes during the period of 'bulk' alveolarization (comparison between 4- and 14-day-old age moments), suggests that this sialic acid is substantially added onto epithelial cell surfaces during this important period of lung development and alveoli formation.

In conclusion, the increasing trend in the levels of Neu5Ac across the developmental relevant age-moments suggests that the surface anionic "shield" created by this sialic acid, and the induced repulsive effect, may be a contributing factor in postnatal alveolar formation and in the maintenance of lung architecture. However, further studies are needed, namely more experiments in the reported ages and quantification of sialic acids at other age-moments.

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References

- Cardoso WV. Lung morphogenesis revisited: old facts, current ideas. *Develop Dynam* 2000;219:121-30.
- Cardoso WV, Lü J. Regulation of early lung morphogenesis: questions, facts and controversies. *Development* 2006;133:1611-24.
- Massaro GD, Massaro D. Postnatal lung growth: evidence that the gas-exchange region grows fastest at the periphery. *Am J Physiol* 1993;265 L319-22.
- Massaro GD, Massaro D. Formation of pulmonary alveoli and gas-exchange surface area: quantification and regulation. *Ann Rev Physiol* 1996;58:73-92.
- McGowan ES, Snyder JM. Development of alveoli. In: Harding R, Pinkerton KE, Plopper CG, editors. *The lung development, aging and the environment*. London: Elsevier Academic Press; 2004. p. 55-73.
- Hameed A, Sherkheli MA, Hussain A, Ul-haq R. Molecular and physiological determinants of pulmonary developmental biology: a review. *Am J Biomed Res* 2013;1:13-24.
- Tschanz SA, Salm LA, Roth-Kleiner M, Barré SF, Burri PH, Schittney JC. Rat lungs show a biphasic formation of new alveoli during postnatal development. *J Appl Physiol*. 2014;117:89-95.
- Burri PH. Structural aspects of postnatal lung development – alveolar formation and growth. *Biol Neonate* 2006;89:313-22.
- Cardoso WV, Whitsett JA. Resident cellular components of the lung. *Proc Am Thorac Soc* 2008;5:767-71.
- Hogan BLM, Barkauskas CE, Chapman HA, Epstein JA, Jain R, Hsia CC, et al. Repair and regeneration of the respiratory system: complexity, plasticity and mechanism of lung stem cell function. *Cell Stem Cell* 2014;15:123-38. <https://www.ncbi.nlm.nih.gov/pubmed/25105578> doi: 10.1016/j.stem.2014.07.012
- Varki A. Biological roles of glycans. *Glycobiology* 2017;27:3-49.
- Haltiwanger RS, Lowe BL. Role of glycosylation in development. *Ann Rev Biochem* 2004;73:491-537.
- Freeze HH, Baum L, Varki A. Glycans in systemic physiology. In: Consortium of Glycobiology, Editors. *Essentials of Glycobiology*. 3rd ed. NY: Cold Spring Harbor Laboratory Press; 2017. p. 521-6.
- Springer SA, Gagneux P. Glycan evolution in response to collaboration, conflict, and constraint. *J Biol Chem* 2013; 288:6904-11.
- Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell* 2006;126:855-67.
- Stanley P, Cummings RD. Structures common to different glycans. In: Consortium of Glycobiology, Editors. *Essentials of Glycobiology*. 3rd ed. NY: Cold Spring Harbor Laboratory Press. p. 161-78.
- Schauer R, Kelm S, Reuter G, Roggentin P, Shaw L. Biochemistry and role of sialic acids. In: Rosenberg A, editor. *Biology of the sialic acids*. NY: Plenum Press; 1995. p. 7-67.
- Varki A, Schnaar RL, Schauer R. 2017. Sialic acids and other nonulosonic acids. In: Consortium of Glycobiology, Editors. *Essentials of Glycobiology*. 3rd ed. NY: Cold Spring Harbor Laboratory Press; 2017. p. 179-96.
- Varki A. Sialic acids in human health and disease. *Cell* 2008;14:353-360.
- Varki A. Are humans prone to autoimmunity? Implications from evolutionary changes in hominin sialic acid biology. *J Autoimm* 2017;83:134-42.
- Bauer J, Osborn HM. Sialic acids in biological and therapeutic processes- opportunities and challenges. *Future Med Chem* 2015;7:2285-99.
- Belardi B, Bertozzi CR. Chemical lectinology: Tools for probing the ligands and dynamics of mammalian lectins in vivo. *Chem Biol* 2015;22:983-93.
- Mulloy B, Dell A, Stanley P, Prestegard JH. Structural analysis of glycans. In: Consortium of Glycobiology, Editors. *Essentials of Glycobiology*. 3rd ed. NY: Cold Spring Harbor Laboratory Press; 2017. p. 639-52.
- Cummings RD, Darvill AG, Etzler ME, Hahn MG. Glycan-recognizing probes as tools. In: Consortium of Glycobiology, Editors. *Essentials of Glycobiology*. 3rd ed. NY: Cold Spring

- Harbor Laboratory Press; 2017. p. 611-25.
25. Lamari FN, Kuhn R, Karamanos NK. Derivatization of carbohydrates for chromatographic, electrophoretic and mass spectrometric structural analysis. *J. Chromatogr B Analyt Technol Biomed Life Sci* 2003;793:15-36.
 26. Martins MF, Bairos V. Glycocalyx of lung epithelial cells. *Int Rev Cytol* 2002;216:132-62.
 27. Ji S, Wang F, Chen Y, Yang C, Zhang P, Zhang X, et al. Developmental changes in the level of free and conjugated sialic acids, Neu5Ac, Neu5Gc and KDN in different organs of pig: a LC-MS/MS quantitative analyses. *Glycoconj J* 2017; 34:21-30.
 28. Martins MF, Honório-Ferreira A, Martins P, Gonçalves C. Presence of sialic acids in bronchioloalveolar cells and identification and quantification of N-acetylneuraminic and N-glycolylneuraminic acids in the lung. *Acta Histochem.* 2019;121:712-7.
 29. Martins MF, Martins P, Gonçalves CA. Presence of N-acetylglucosamine residues on the surface coating of bronchioloalveolar cells during rat postnatal development: what is their purpose? *Acta Histochem* 2018;121:119-24.
 30. Maeda T, Balakrishnan K, Mehdi SQ. A simple and rapid method for the preparation of plasma membranes. *Biochim Biophys Acta* 1983;731:115-20.
 31. Anumula KR. Rapid quantitative determination of sialic acids in glycoproteins by High-Performance Liquid Chromatography with sensitive fluorescence detection. *Anal Biochem* 1995;230:24-30.
 32. Gonçalves CA, Barros J, Honório A, Rodrigues P, Bairos V. 2001. Quantification of elastin from mouse lung during postnatal development. *Exp Lung Res* 2001;27:533-45.
 33. Morrisey EE, Hogan BLM. Preparing for the first breath: Genetic and cellular mechanisms in lung development. *Dev Cell* 2010;18:8-23.
 34. Li F, Ding J. Sialylation is involved in cell fate decision during development, reprogramming and cancer progression. *Protein Cell* 2019;10:550-65.
 35. Pierce M, Stanley P. Deuterostomes. In: Consortium of Glycobiology, Editors. *Essentials of Glycobiology*. 3rd ed. NY: Cold Spring Harbor Laboratory Press; 2017. p. 351-60.
 36. Buttner B, Kannicht C, Schmidt C, Loster K, Reutter W, Lee H-Y, et al. Biochemical engineering of cell surface sialic acids stimulate axonal growth. *J Neurosci* 2002;22:8869-75.
 37. Wang B. Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Adv Nutr.*2012;3:465S-72.
 38. Schnaar RL, Gerardy-Schahn R, Hildebrandt H. Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration. *Physiol Rev* 2014;94:461-518.
 39. Muramatsu T. Essential roles of carbohydrate signals in development, immune response and tissue functions, as revealed by gene targeting. *J Biochem* 2000;127:171-6.
 40. Weinhold B, Sellmeier M, Schaper W, Blume L, Philippens B, Kats E, et al. Deficits in sialylation impair podocyte maturation. *J Am Soc Nephrol* 2012;23:1319-28.
 41. Minoo P. Transcriptional regulation of lung development: emergence of specificity. *Resp Res.*2000;1:109-15.
 42. Cerná A, Janega P, Martanovic P, Lisý M, Babál P. Changes in sialic acid expression in the lung during intrauterine development of human fetus. *Acta Histochem* 2002;10:339-42.
 43. Martins MF, Martins P, Gonçalves CA. Presence of N-acetylgalactosamine/galactose residues on bronchioloalveolar cells during rat postnatal development. *Eur J Histochem* 2019;63:3040.
 44. Schittny JC. Development of the lung. *Cell Tissue Res* 2017;367:427-44.
 45. Schittny JC. How high resolution 3-dimensional imaging changes our understanding of postnatal lung development. *histochemistry and cell biology.* 2018;150:677-91.
 46. Burri PH, Dbaly J, Weibel ER. The postnatal growth of the rat lung I. Morphometry. *Anat Rec* 1974;178:711-30.
 47. Burri PH. The postnatal growth of the rat lung III. Morphometry. *Anat Rec* 1974;180:77-98.
 48. Burri PH. Postnatal growth and maturation of the lung. *Chest* 1975;67:2S-3.
 49. Massaro D, Teich N, Maxwell S, Massaro GD, Whitney P. Postnatal development of alveoli. *J Clin Invest* 1985;76:1297-305.
 50. Schittny JC, Mund SI, Stampanoni M. Evidence and structural mechanism for late lung alveolarization. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L246-54.
 51. Barré SF, Haberthur D, Cremona TP, Stampanoni M, Schittny JC . The total number of acini remains constant throughout postnatal rat lung development. *Am J Physiol Lung Cell Mol Physiol* 2016;311:L1082-9.
 52. Barré SF, Haberthur D, Stampanoni M, Schittny JC. Efficient estimation of total number of acini in adult rat lung. *Physiol Rep* 2014;2:e12063.
 53. Lim S, Kemmner W, Grigull S, Schlag PM. Cell surface alpha 2,6 sialylation affects adhesion of breast carcinoma cells. *Exp Cell Res* 2002;276:101-10.
 54. French BM, Sendil S, Pierson III RN, Azimzadeh AM. The role of sialic acids in the immune recognition of xenografts. *Xenotransplantation* 2017;24. doi: 10.1111/xen.12345
 55. Nigam PK, Narain VS, Kumar A. Sialic acid in cardiovascular diseases. *Indian J Clin Biochem* 2006;21:54-61.
 56. Wang X, Li X, Zeng Y-N, He F, Yang X-M, Guan F. Enhanced expression of polysialic acid correlates with malignant phenotype in breast cancer cell lines and clinical tissue samples. *Int J Mol Med* 2016;37:197-206.

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