Neuroendocrine Cell Distribution and Frequency Distinguish Neuroendocrine Cell Hyperplasia of Infancy From Other Pulmonary Disorders

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Background

The diagnostic gold standard for neuroendocrine cell hyperplasia of infancy (NEHI) is demonstration of increased numbers of neuroendocrine cells (NECs) amid otherwise near-normal lung histology. Typical clinical and radiographic features often are present. However, NECs are also increased after lung injury and in other disorders, which can complicate biopsy specimen interpretation and diagnosis of suspected NEHI. Our objective was to determine whether NEC prominence is specific for the diagnosis of NEHI.

Methods

Bombesin immunoreactivity was quantified in lung biopsy specimens from 13 children with characteristic clinical presentation and imaging appearance of NEHI. The primary comparison group was 13 age-matched patients selected from children with lung disorders that are known to be associated with NEC prominence.
Results
Bombesin-immunopositive epithelial area was significantly increased in NEHI compared with other diseases. Patchy bronchiolar inflammation or fibrosis was frequently observed in NEHI, with no direct association between airway histopathology and bombesin-immunopositive area. NEC prominence correlated with severity of small airway obstruction demonstrated on infant pulmonary function testing. Immunohistochemical colocalization of bombesin with Ki67 did not reveal active NEC proliferation. There was wide intra- and intersubject variability in NEC number, which did not relate to radiographic appearance of the region biopsied.

Conclusions
Our findings demonstrate that NEC prominence is a distinguishing feature of NEHI independent of airway injury. The extent of intrasubject variability and potential for overlap with control subjects suggest that clinical-radiologic-pathologic correlation is required for diagnosis and that the abundance of NECs may not fully explain the disease pathogenesis.

Abbreviations
BPD
bronchopulmonary dysplasia

FEF
forced expiratory flow

FEV0.5
forced expiratory volume in 0.5 s

FRC
functional residual capacity

HRCT
high-resolution CT

NEB
neuroepithelial body

NEC
neuroendocrine cell

NEHI
neuroendocrine cell hyperplasia of infancy

PFT
pulmonary function test

RV
residual volume

TLC
total lung capacity

Neuroendocrine cell hyperplasia of infancy (NEHI) is a poorly understood pulmonary disorder. Patients typically present in the
first year of life with chronic tachypnea, retractions, crackles, and hypoxia. They have no sustained response to systemic corticosteroids or bronchodilators. High-resolution CT (HRCT) scanning shows geographic ground-glass opacities commonly involving the right middle lobe and lingula and other large areas of relative hyperlucency with air trapping. Although the sensitivity of HRCT scanning for use in the diagnosis of NEHI is incomplete (78%), high specificity has been reported.

Lung biopsy specimens in NEHI were originally described as normal or with minor nonspecific changes. The only consistent abnormality seen in lung biopsy specimens is an increased proportion of neuroendocrine cells (NECs) within distal airways best demonstrated by bombesin and serotonin immunohistochemistry. There are no formal criteria for defining NEC excess in the lung, but it has been suggested that findings of NECs within ≥ 70% of bronchioles in the lung biopsy specimen and ≥ 10% NECs in an individual airway are consistent with the diagnosis in the appropriate clinical setting.

Pulmonary NECs, which produce bioactive products, including bombesin-like peptide and serotonin, are specialized epithelial cells scattered throughout the conducting airways and as innervated clusters (neuroepithelial bodies [NEBs]). In the fetus, NECs are most abundant in the distal airways where they promote branching morphogenesis, epithelial and mesenchymal cell proliferation, and surfactant secretion. Postnatally, NECs function as oxygen chemosensors and degranulate in response to hypoxia. Although NECs decline rapidly in number after the neonatal period, NEC hyperplasia has been described in a number of conditions or disorders, including bronchopulmonary dysplasia (BPD), sudden infant death syndrome, pulmonary hypertension, cystic fibrosis, and mechanical ventilation. Increased NEC number with proliferation also has been observed in animal models of airway epithelial repair, imparting a progenitor cell role for NECs.

Increasing clinical experience with NEHI has resulted in the recognition of a wider range of pathologic features than the near-normal appearance previously described, most commonly, patchy airway inflammation or fibrosis. Although NECs have been shown to be increased in patients with NEHI compared with age-matched control subjects, there has been no systematic comparison with other pulmonary disorders associated with NEC hyperplasia. The aim of this study was to determine whether there are quantifiable differences in the abundance and pattern of NECs in patients with NEHI vs an age-matched disease cohort comprising patients with disorders associated with increased NECs or airway injury. Our hypothesis was that NEC prominence in NEHI would be distinct and not explained by airway injury.

Materials and Methods

Study Population

After Institutional Review Board approval, all lung biopsies performed at Cincinnati Children's Hospital Medical Center from 1999 to 2008 for diffuse lung disease or that were reviewed in both clinical and pathology consultation (N = 138) were screened for possible study inclusion.

NEHI Group

Because a study objective was to define the histologic spectrum, stringent clinical and radiographic inclusion criteria were used for the diagnosis of NEHI. Specifically, subjects with NEHI were term or near-term infants with indolent onset of chronic tachypnea, retractions, and hypoxemia in the first year of life. Chest HRCT scan obtained nearest in time to lung biopsy was reviewed by a radiologist who was blinded to clinical information and numbers of cases in each study group. Inclusion criteria for NEHI required the HRCT scan to be rated on a 5-point Likert scale as strongly agree or agree with diagnosis of NEHI based on radiographic features previously reported. In total, 19 cases of NEHI were identified; four were excluded because either the lung biopsy specimens lacked a minimum of 10 airways for evaluation or tissue blocks were not available. Two other potential patients with NEHI were excluded because they did not meet the prespecified clinical and radiographic criteria. Thus, 13 subjects comprised the NEHI group. Independent case review was performed by two pediatric pulmonary pathologists.

Other-Diseases Comparison Group and Control Group

For the other-diseases group, comparison cases were selected to achieve a balanced sample size age matched with the NEHI cases and to include other pulmonary disorders associated with NEC hyperplasia (bronchiolitis, BPD, pulmonary hypertension). As a control group, uninvolved lung from lobectomy cases for congenital cystic adenomatoid malformations served as an additional comparison (n = 6).

Infant Pulmonary Function Tests

Infant pulmonary function tests (PFTs) were performed as part of clinical care for some of the subjects in these cohorts.
Testing was performed with oral chloral hydrate sedation (75-100 mg/kg) using the nSpire Infant Pulmonary Laboratory system and its updated integrated software (vCM55Ra; nSpire; Longmont, Colorado). After static measures, plethysmography was performed as described by Castile et al.,[22] and then flow-volume curves were obtained using the raised-volume rapid thoracoabdominal compression technique described by Jones et al.[23] Studies were performed and interpreted according to American Thoracic Society/European Respiratory Society guidelines.[24]

**Immunohistochemistry**

To delineate NECs, immunohistochemistry for bombesin (ImmunoStar, Inc; Hudson, Wisconsin) was performed on formalin-fixed 5-µm paraffin sections as previously described.[2] Dual immunofluorescence for bombesin with Ki67 (1:100) (Dako M7249; Dako North America; Carpinteria, California) was carried out on paraffin sections following antigen retrieval. Bombesin was developed with antirabbit fluorescein and Ki67 with biotinylated antimouse IgG (both from Vector Laboratories Inc; Burlingame, California) followed by Cy3-streptavidin (Jackson ImmunoResearch; West Grove, Pennsylvania).

**Morphometric Analysis**

Morphometry was performed on sections immunostained for bombesin similar to that previously described,[2] using images captured by a SPOT RT Slider digital camera (Diagnosics Instruments, Inc; Sterling Heights, Michigan) mounted on a Nikon E400 microscope (Nikon Instruments Inc; Melville, New York). Digital images of all peripheral (noncartilage-containing) airways were captured using the 20 × objective, and airway epithelium and bombesin-immunopositive areas were outlined manually, with area measurements expressed as square micrometers using SPOT Advanced software version 4.6 (Diagnosics Instruments, Inc). The investigator performing morphometric analyses was blinded to case identification. Airways were classified as either proximal (comprising membranous and terminal bronchioles) or respiratory (air passages comprising part muscular bronchial wall and part alveoli) bronchioles, and the presence of inflammatory or remodeling changes were documented. In each airway, the immunostained area was expressed as a percentage of the total airway epithelial area (%NEC area). The area of bombesin-immunopositive NEBs in the alveolar ducts was divided by the area of the tissue section to obtain %NEB area. For subjects with more than one biopsy site (subjects 2 and 6-10), quantification of bombesin-immunopositive area was analyzed as the aggregate of all airways counted.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism and InStat (GraphPad Software, Inc; San Diego, California). Data were compared with Mann-Whitney U test or Kruskal-Wallis with Dunn multiple comparisons test. Association between age and NEC indices was measured using Spearman correlation. A two-sided P < .05 was regarded as significant.

**Results**

**Clinical, Radiographic, and Histologic Features of Study Subjects**

Table 1 shows the overall demographic and clinical features of the study population. Prominent comorbidities were present in the other-diseases cohort as anticipated by the study design. Characteristic chest HRCT scans and histology from two subjects with NEHI are shown in Figure 1 compared with a patient with bronchiolitis obliterans (Figs 1A-F). Table 2 details the histologic findings in each patient. The initial pathologic interpretation of increased pulmonary NECs was based on the previously proposed criteria.[5] In eight cases, either some airway inflammation or fibrosis was present, albeit involving a small proportion of airways (Fig 1E). Four subjects with NEHI had long-standing tachypnea and retractions for which evaluation was ongoing but then experienced confirmed intercurrent viral infection prior to biopsy.

**Table 1 -- Demographic and Clinical Features of the Study Population**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>NEHI Group (n = 13)</th>
<th>Other-Diseases Group (n = 13)</th>
<th>Control Subjects (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>7 (54)</td>
<td>7 (54)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>Age at biopsy, mo</td>
<td>12.1 ± 19.7 (9.5)[a]</td>
<td>17.0 ± 15.4 (13.3)</td>
<td>11.6 ± 11.7 (8.2)</td>
</tr>
<tr>
<td>Clinical history and comorbidities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preterm birth</td>
<td>1 (8)[b]</td>
<td>5 (38)</td>
<td>0</td>
</tr>
<tr>
<td>Supplemental oxygen at birth</td>
<td>0</td>
<td>6 (55)</td>
<td>0</td>
</tr>
<tr>
<td>Congenital heart disease</td>
<td>1 (8)</td>
<td>6 (46)</td>
<td>0</td>
</tr>
<tr>
<td>Characteristic</td>
<td>NEHI Group (n = 13)</td>
<td>Other-Diseases Group (n = 13)</td>
<td>Control Subjects (n = 6)</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------</td>
<td>---------------------</td>
<td>-------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Immunodeficiency</td>
<td>0</td>
<td>2 (15)</td>
<td>0</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>10 (77)</td>
<td>10 (77)</td>
<td>0</td>
</tr>
<tr>
<td>Supplemental enteral feeds</td>
<td>5 (38)</td>
<td>7 (54)</td>
<td>0</td>
</tr>
<tr>
<td>Radiographic appearance rated as suggestive of NEHI [c]</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strongly agree</td>
<td>8</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Agree</td>
<td>5</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Neutral</td>
<td>0</td>
<td>1</td>
<td>...</td>
</tr>
<tr>
<td>Disagree</td>
<td>0</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Strongly disagree</td>
<td>0</td>
<td>8</td>
<td>...</td>
</tr>
<tr>
<td>Hypoxemia or supplemental oxygen use at time of biopsy</td>
<td>13 (100)</td>
<td>11 (85)</td>
<td>0</td>
</tr>
<tr>
<td>Lung transplantation or mortality</td>
<td>0</td>
<td>4 (31)</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are presented as No. (%), mean ± SD (median), counts. n/a = not applicable; NEHI = neuroendocrine cell hyperplasia of infancy.

**a**  
P not significant for the NEHI group vs the other-diseases group and the control group.

**b**  
One subject was born at 35.5 wk estimated gestational age, had no perinatal respiratory symptoms, and did not require supplemental oxygen in the neonatal period.

**c**  
Chest CT scans were not performed in four of the 13 other-diseases patients.
Figure 1  Representative radiographic and histologic findings of study subjects. A-F, Findings in subjects with neuroendocrine cell hyperplasia of infancy (NEHI). A, D, High-resolution CT scan reveals prominent ground-glass attenuation in the right middle lobe and lingula in two subjects with NEHI. In comparison to an 8-month-old subject with normal lung histology (B and C, original magnification × 200), there was patchy airway injury in the lung biopsy specimen from subject 5 (age, 6.5 months) who had otherwise well-preserved bronchioles with prominent clusters of bombesin immunopositive cells (E and F, original magnification × 100). G-I, Radiographic and histologic findings in an other-diseases group subject with bronchiolitis obliterans. High-resolution CT scan demonstrates areas of mosaic areas of decreased attenuation and bronchiectasis, and the lung biopsy specimen demonstrates prominent bronchiolar remodeling with airway constriction. Hematoxylin and eosin was used in B and E; bombesin stain was used in C, F, and I; Movat pentachrome stain (× 200) was used in H.

Table 2  -- Subject Histologic Diagnoses and Outcomes

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age at Biopsy, y</th>
<th>Primary Histologic Diagnosis</th>
<th>Secondary Histologic Findings</th>
<th>Pathogens Identified</th>
<th>Other Clinical History</th>
<th>Age at O₂ Liberation, y</th>
<th>Follow-up Status</th>
<th>Follow-up Age, y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>NEHI</td>
<td>Patchy chronic bronchiolitis</td>
<td>...</td>
<td>...</td>
<td>8</td>
<td>Asymptomatic</td>
<td>13.8</td>
</tr>
<tr>
<td>2</td>
<td>3.6</td>
<td>NEHI</td>
<td>Patchy chronic bronchiolitis</td>
<td>...</td>
<td>...</td>
<td>6</td>
<td>Symptomatic, no O₂</td>
<td>8.0</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>NEHI</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>n/a</td>
<td>Continuous O₂</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>1.1</td>
<td>NEHI</td>
<td>...</td>
<td>Adenovirus BAL</td>
<td>Bronchiolitis 2 mo prior[a]</td>
<td>2.2</td>
<td>Asymptomatic</td>
<td>4.3</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>NEHI</td>
<td>Patchy chronic bronchiolitis</td>
<td>...</td>
<td>RSV 2 mo prior[a]</td>
<td>n/a</td>
<td>O₂ sleep only</td>
<td>3.3</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>NEHI</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>n/a</td>
<td>Continuous O₂</td>
<td>1.5</td>
</tr>
<tr>
<td>7</td>
<td>0.7</td>
<td>NEHI</td>
<td>Patchy chronic bronchiolitis</td>
<td>...</td>
<td>Parainfluenza Bronchiolitis 1 mo prior[a]</td>
<td>n/a</td>
<td>O₂ sleep/exercise</td>
<td>3.0</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>NEHI</td>
<td>Patchy chronic bronchiolitis</td>
<td>...</td>
<td>...</td>
<td>1.5</td>
<td>Symptomatic, no O₂</td>
<td>2.8</td>
</tr>
<tr>
<td>9</td>
<td>2.1</td>
<td>NEHI</td>
<td>Patchy chronic bronchiolitis</td>
<td>...</td>
<td>...</td>
<td>n/a</td>
<td>O₂ sleep only</td>
<td>3.8</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>NEHI</td>
<td>Aspiration[b]</td>
<td>...</td>
<td>Aspiration, RSV 4 mo prior[a]</td>
<td>n/a</td>
<td>O₂ sleep/exercise</td>
<td>2.0</td>
</tr>
<tr>
<td>11</td>
<td>0.8</td>
<td>NEHI</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>n/a</td>
<td>O₂ sleep only</td>
<td>2.6</td>
</tr>
<tr>
<td>12</td>
<td>1.2</td>
<td>NEHI</td>
<td>PHTN[c]</td>
<td>...</td>
<td>PHTN</td>
<td>n/a</td>
<td>Continuous O₂</td>
<td>2.3</td>
</tr>
<tr>
<td>13</td>
<td>0.5</td>
<td>NEHI</td>
<td>Patchy chronic bronchiolitis</td>
<td>...</td>
<td>...</td>
<td>n/a</td>
<td>O₂ sleep only</td>
<td>2.2</td>
</tr>
<tr>
<td>14</td>
<td>0.3</td>
<td>PHTN</td>
<td>Patchy pulmonary interstitial glycogenosis</td>
<td>...</td>
<td>TAPVR, pulmonary vein stenosis</td>
<td>0.4</td>
<td>Symptomatic, no O₂</td>
<td>4.5</td>
</tr>
<tr>
<td>15</td>
<td>3.3</td>
<td>Acute and chronic bronchiolitis with constriction</td>
<td>...</td>
<td>...</td>
<td>n/a</td>
<td>Lung transplantation</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.8</td>
<td>BPD/PHTN</td>
<td>...</td>
<td>...</td>
<td>33-wk EGA, VSD, Trisomy 21</td>
<td>n/a</td>
<td>Death</td>
<td>1.3</td>
</tr>
</tbody>
</table>
### Extent and Anatomic Distribution of NECs

Biopsy specimens from the NEHI group, other-diseases group, and control group all had some bombesin-immunopositive cells and similar intensity of bombesin staining (Figs 2A-F). All subjects with NEHI had NECs in > 70% of bronchioles, although five of the 13 other-diseases subjects and five of the six control subjects also met this criterion. All subjects in the NEHI group and 50% of those in the other-diseases group also had at least one individual airway with ≥ 10% NECs, suggesting that these histologic criteria alone are sensitive but not specific for the diagnosis of NEHI.

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<table>
<thead>
<tr>
<th>Subject</th>
<th>Age at Biopsy, y</th>
<th>Primary Histologic Diagnosis</th>
<th>Secondary Histologic Findings</th>
<th>Pathogens Identified</th>
<th>Other Clinical History</th>
<th>Age at O2 Liberation, y</th>
<th>Follow-up Status</th>
<th>Follow-up Age, y</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>0.8</td>
<td>Acute bronchiolitis with organizing pneumonia/BPD</td>
<td>...</td>
<td>RSV</td>
<td>28-wk EGA, RSV 2 mo prior</td>
<td>n/a</td>
<td>Asymptomatic</td>
<td>1.8</td>
</tr>
<tr>
<td>18</td>
<td>1.5</td>
<td>PHTN</td>
<td>...</td>
<td>...</td>
<td>TAPVR</td>
<td>n/a</td>
<td>Asymptomatic</td>
<td>2.7</td>
</tr>
<tr>
<td>19</td>
<td>1.7</td>
<td>Lingular bronchiolitis</td>
<td>...</td>
<td>EBER positive</td>
<td>DiGeorge, prior RSV, aspiration</td>
<td>n/a</td>
<td>Symptomatic, no O2</td>
<td>7.1</td>
</tr>
<tr>
<td>20</td>
<td>4.7</td>
<td>Necrotizing bronchiolitis</td>
<td>...</td>
<td>RSV, Pneumocystis</td>
<td>SCID</td>
<td>n/a</td>
<td>Death</td>
<td>4.8</td>
</tr>
<tr>
<td>21</td>
<td>1.0</td>
<td>BPD</td>
<td>Bronchiolitis</td>
<td>...</td>
<td>31-wk EGA, remote influenza and RSV</td>
<td>2</td>
<td>Asymptomatic</td>
<td>5.0</td>
</tr>
<tr>
<td>22</td>
<td>0.4</td>
<td>BPD/PHTN</td>
<td>...</td>
<td>...</td>
<td>Hypoplastic left heart</td>
<td>n/a</td>
<td>Symptomatic, no O2</td>
<td>3.8</td>
</tr>
<tr>
<td>23</td>
<td>1.0</td>
<td>PHTN</td>
<td>...</td>
<td>...</td>
<td>Complex heart disease</td>
<td>n/a</td>
<td>Death</td>
<td>1.1</td>
</tr>
<tr>
<td>24</td>
<td>1.8</td>
<td>Chronic bronchiolitis</td>
<td>...</td>
<td>...</td>
<td>Adenovirus 1 mo prior, chronic spiration</td>
<td>2</td>
<td>Symptomatic, no O2</td>
<td>2.3</td>
</tr>
<tr>
<td>25</td>
<td>0.03</td>
<td>Pulmonary interstitial glycogenosis</td>
<td>PHTN</td>
<td>...</td>
<td>Aortic coarctation</td>
<td>0.4</td>
<td>Asymptomatic</td>
<td>2.5</td>
</tr>
<tr>
<td>26</td>
<td>1.1</td>
<td>BPD</td>
<td>Bronchiolectasis, PHTN</td>
<td>...</td>
<td>30-wk EGA</td>
<td>n/a</td>
<td>Continuous O2, PHTN</td>
<td>3.5</td>
</tr>
</tbody>
</table>

BPD = bronchopulmonary dysplasia; EBER = Epstein-Barr virus-encoded small RNA; EGA = estimated gestational age; O2 = oxygen; PHTN = pulmonary hypertension, RSV = respiratory syncytial virus, SCID = severe combined immunodeficiency; TAPVR = total anomalous pulmonary venous return; VSD = ventricular septal defect. See Table 1 legend for expansion of other abbreviations.

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**a** These four subjects had longstanding tachypnea and retractions of indolent onset, and all were already undergoing medical evaluations for their chronic symptoms prior to the onset of a viral illness.

**b** Four months after initial presentation with chronic tachypnea and retractions, this subject with NEHI contracted RSV bronchiolitis and then developed feeding incoordination, with aspiration documented on fluoroscopic swallow evaluation. Histologic evidence of aspiration was based on the presence of intraairway proteinaceous debris and multinucleated macrophages.

**c** This subject with NEHI had PHTN documented by cardiac catheterization. Medial thickening of small pulmonary arteries and muscularization of intralobular vessels were seen in the lung biopsy specimen.
Figure 2  Neuroendocrine cell (NEC) prominence and distribution distinguishes NEHI from other disorders. A, Schematic diagram of the components of proximal and distal airspaces. B-F, Within NEHI, NECs are more prominent in respiratory bronchioles (D, × 100) and in the presence of neuroepithelial bodies (NEBs) around alveolar ducts (F, × 200) than in more proximal bronchioles (B and C, × 200), where the presence of NECs is more variable. E, Respiratory bronchiole in an other-diseases group patient for comparison (× 200). G, Group means and quantitation of the %NEC area (% bombesin-positive epithelial area) in individual NEHI subjects vs other-diseases subjects and control subjects (*\( P < .001 \) for NEHI vs other-diseases group and control subjects; \( P \) not significant for other-diseases group vs control subjects). H, Quantitation of the %NEC area for all cases based on anatomic location within either proximal or respiratory bronchioles (*\( P < .05 \) for the NEHI group vs the other-diseases group in the proximal bronchioles; **\( P < .001 \) for NEHI vs other-diseases group; \( P < .01 \) vs control subjects in the more distal respiratory bronchioles). I, %NEB area was also significantly greater in NEHI cases than in other disease cases (*\( P < .001 \) or control subjects (*\( P < .05 \)). AD = alveolar duct; AS = alveolar sac; RB = respiratory bronchiole; TB = terminal membranous bronchiole. See Figure 1 legend for expansion of the other abbreviation.

In contrast, quantitative morphometric analysis of the total %NEC area showed a threefold greater NEC presence in subjects with NEHI vs other-diseases subjects and control subjects (Fig 2G). Although one patient in the other-diseases group (subject 21) had elevated total %NEC area, the 95% CIs of %NEC area were nonoverlapping between the NEHI and other-diseases group (95% CI, 4.5% to 6.4% vs 1.1% to 2.3%, respectively). In 15 cases (seven NEHI subjects and eight other-diseases subjects), the number of immunopositive cells also was determined by cell counting and analyzed as a percentage of the total number of bronchiolar cells (% bombesin-positive epithelial cells). This alternative methodologic approach of cell counting also confirmed an increase in NECs (NEHI group, 5.13% ± 1.58%; other-diseases group, 1.88% ± 1.31%; \( P < .01 \); not shown). There was a strong correlation in extent of NEC prominence based on the two analysis methods (Spearman \( r = 0.85 \)).

As shown in Figure 2H, the %NEC area was approximately twofold greater in the proximal bronchioles of NEHI vs other-diseases cases, although overlapping values were observed. In the distal respiratory bronchioles, subjects with NEHI demonstrated a marked increase in %NEC area. The %NEB area was also significantly greater in subjects with NEHI, but again, overlapping values were observed (Fig 2I).

**Infant PFT Abnormalities in Subjects With NEHI**

We used infant PFTs ([22] , [23]) to examine the relationship between NEC prominence and pulmonary physiology in NEHI. Ten subjects with NEHI had infant PFT studies that met American Thoracic Society/European Respiratory Society criteria for
acceptable and repeatable measures of forced expiratory flows (FEFs) and volumes using the raised-volume rapid thoracoabdominal compression technique\[24] (Table 3). One subject had not undergone testing, and two had infant PFTs at other centers, the results of which were excluded because we could not review the primary data for quality control. As previously recognized, subjects with NEHI showed a mixed physiologic pattern, including profound air trapping.\[25] There were proportionate reductions in the forced expiratory volume in 0.5 s and FVC, with particularly reduced FEF at 75% and 85% of FVC, and markedly elevated functional residual capacity (FRC), residual volume (RV), and RV/total lung capacity (TLC) ratio. In patients with NEHI, increased extent of %NEC area correlated with a greater degree of reduction of small airway flows based on FEF at 75% of FVC \( (r = -0.73; P < .05) \) and FEF at 85% of FVC \( (r = -0.726; P < .05) \). There was no significant association between NEC area and FEF at 25% of FVC \( (r = -0.323) \), FEF at 50% of FVC \( (r = -0.564) \), RV \( (r = 0.261) \), RV/TLC \( (r = -0.382) \), or FRC \( (r = 0.212) \).

Table 3 -- Infant Pulmonary Function Test Data on Subjects With NEHI

<table>
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<th>Subject Age, mo</th>
<th>FEV0.5/FVC %</th>
<th>FEV0.5 %</th>
<th>FVC %</th>
<th>FEF25</th>
<th>FEF50</th>
<th>FEF75</th>
<th>FEF85</th>
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Data are percentages of predicted based on normative data.\[22]\ .\[23\] FEF25 = forced expiratory flow at 25% of FVC; FEF50 = forced expiratory flow at 50% of FVC; FEF75 = forced expiratory flow at 75% of FVC; FEF85 = forced expiratory flow at 85% of FVC; FEF25–75 = forced expiratory flow at 25% to 75% of FVC; FEV0.5 = forced expiratory volume in 0.5 s; FRC = functional residual capacity; IQR = interquartile range; RV = residual volume; TLC = total lung capacity. See Table 1 legend for expansion of other abbreviation.

Relationship Between NEC Prominence and Radiographic Appearance

We sought to determine whether there were regional differences in NEC number in ground-glass regions compared with hyperlucent regions. Six subjects with NEHI had biopsy specimens taken from more than one region, with locations of ground glass and hyperlucency guided by the HRCT scan appearance (Fig 3A). There was significant intrasubject variation in the %NEC and %NEB areas, without apparent relationship to the radiographic appearance of the region from which the biopsy specimen was removed (Figs 3B, 3C). Further, when compared with control cases, several of the NEHI biopsy specimens showed no increase in NECs or NEBs, indicating that histologic diagnosis would not have been confirmed without the second biopsy site in these patients (ie, subject 8). These findings suggest that ground-glass appearance is not explained by the histology and number of NECs.
Figure 3  Imaging findings do not correlate with NEC frequency in NEHI. A, Example of classification of the radiographic appearance of biopsied regions as either ground-glass opacity or normal/hyperlucent. B and C, There is wide intersubject and intrasubject variability in the % NEC area and % NEB area, respectively, in each biopsy specimen by individual subject. Six of the 13 subjects had multiple biopsy sites, including two who had three biopsy sites (subjects 6 and 8). The regions with ground-glass opacity were in the right middle lobe or lingula in all subjects, whereas the normal/hyperlucent regions were sampled from other geographic regions. All subjects with only a single biopsy site had biopsy specimens obtained from the right middle lobe or lingula. The dashed lines indicate the 95% CI of the upper limit of normal area in control subjects. See [Figure 1], [Figure 2] legends for expansion of other abbreviations.

Airway Injury in Subjects With NEHI

We analyzed the extent of airway injury in patients with NEHI and its relationship to NEC prominence. As shown in Figure 4A, 89.7% of all airways in subjects with NEHI had no histologic injury present, whereas inflammatory or remodeling changes were present in 10% of airways from the aggregate NEHI cohort. In patients with NEHI, NECs were increased specifically in the noninjured airways (Figs 4B, 4E-J). Four subjects with NEHI (4, 5, 7, and 10) (Table 2) had intercurrent viral infection prior to lung biopsy. Others had histologic evidence of minor airway injury without recognized clinical complications. The degree of NECs and NEBs was not significantly different in patients with NEHI with histologic injury compared with those without injury (Fig 4C, 4D). In addition, there was no significant difference in NEC or NEB area between NEHI cases with clinical confounders of aspiration or viral infection and those without these features (%NEC area, 5.74 ± 0.29 vs 5.31 ± 0.64, respectively; P not significant; %NEB area, 700 ± 30.6 × 10⁻⁵ vs 719.4 ± 253.4 × 10⁻⁵, respectively; P not significant). In aggregate, these findings argue against airway injury as an etiology of NEHI. Furthermore, the data support that patchy airway injury in the setting of intercurrent viral infection or aspiration does not exclude the diagnosis of NEHI in the appropriate clinical and radiographic setting.
NEC hyperplasia is present in uninjured airways in NEHI. A, A small proportion of airways are injured in NEHI cases. The other-diseases cohort was intentionally enriched for airway injury. B, NEC prominence in NEHI case is found in noninjured airways (*P < .001 for %NEC area in NEHI airways without injury vs all other groups; P not significant for %NEC area in other-diseases group subjects with airways with and without injury). C and D, The %NEC area and %NEB area, respectively, in individual NEHI cases based on presence or absence of histologic injury. Black symbols denote those cases with proven viral infection near time of biopsy (P not significant for both %NEC area and %NEB area). E-J, Inflamed (E and F, original magnification × 200) and fibrotic (H and I, original magnification × 200) bronchioles in NEHI cases show minimal bombesin-immunopositive cells (arrows) compared with adjacent uninjured airways (G and J, original magnification × 200). See [Figure 1] , [Figure 2] legends for expansion of abbreviations.

**Factors Contributing to NEC Prominence**

NECs have been shown to significantly decline after the neonatal period.[12] In the NEHI cohort in which the earliest age at biopsy was 4.7 months and latest 45 months, there was no correlation between age at biopsy and NEC or NEB area (r = 0.177 and r = 0.254, respectively, data not shown). BPD, pulmonary hypertension, and bronchiolitis, may rarely complicate NEHI; therefore, we analyzed the impact of these features on NEC prominence in the other-diseases cohort. BPD was associated with significantly increased %NEC area, but there was no significant difference in %NEC or %NEB area based on the presence or absence of pulmonary hypertension or bronchiolitis (Fig 5).
Assessment of NEC Proliferation

To determine whether active NEC proliferation was present in NEHI, we performed dual immunofluorescence for bombesin and the proliferative marker Ki67. Although there were proliferating epithelial cells and lymphocytes within bronchioles, no colocalization for bombesin and Ki67 was seen in five patients with NEHI and biopsy specimens from three other-diseases subjects (Fig 6).

Figure 5  NEC prominence is associated with BPD but not pulmonary hypertension or bronchiolitis. Other-diseases cases were classified based on the pathologic findings of BPD, PHTN, or bronchiolitis. A, C, and E, The mean ± SD for %NEC area based on these features. B, D, and F, The %NEB area. *P < .01 for total airway %NEC area, proximal bronchiole %NEC area, and respiratory bronchiole %NEC area for BPD (n = 5) vs non-BPD (n = 8) other-diseases cases. P not significant for %NEB area based on presence or absence of BPD and for %NEC area and %NEB area based on presence or absence of PHTN or bronchiolitis. BPD = bronchopulmonary dysplasia; PHTN = pulmonary hypertension. See Figure 2 legend for expansion of other abbreviations.
Figure 6  NEC prominence is not associated with active cell proliferation. Shown are representative images of dual immunofluorescence for bombesin (green) and Ki67 (red) in subjects with NEHI. Colocalization of bombesin with Ki67 is not seen in any airways (A, × 200) or neuroendocrine bodies (B, × 400), although proliferating cells (arrows) are seen adjacent to bombesin-immunopositive areas. See [Figure 1] , [Figure 2] legends for expansion of abbreviations.

Discussion

The association of NEC hyperplasia with a variety of pulmonary disorders has led to questions of whether NEHI is a primary disease, a reactive feature of airway injury, or hypoxia in the immature lung. Furthermore, because the original description of NEHI was that of near-normal histology, clinical diagnostic dilemmas have emerged in patients when some degree of lung injury is observed. We aimed to address the specificity of NEC prominence in distinguishing NEHI from other pulmonary disorders, particularly those associated with hypoxemia and airway injury. Both morphometric analysis of bombesin immunopositivity and an alternative quantitative method of cell counting confirmed that NEC prominence is a distinguishing feature of NEHI.

In order to define the histologic spectrum of NEHI, study inclusion required highly characteristic clinical and radiographic features of the disorder, and the size of our study cohort was limited by the prevalence of NEHI. However, our NEC area data are consistent with previous reports. Specifically, compared with the publication by Deterding et al, the extent of NEC prominence in NEHI subjects was similar (5.44% vs 6.52%), as was the relative difference between NEHI subjects and control subjects (3.89% vs 3.69%). Additionally, the NEC area in our control subjects was not significantly different than the control data in a larger series by Cutz et al (1.55% ± 0.25% [95% CI, 0.91-2.19] vs 1.88% ± 0.24% [95% CI, 1.38-2.34], respectively; \( P \) not significant).

For relevance with respect to clinical diagnosis and disease mechanisms, the other-diseases cohort was selected based on patients having disorders associated with the highest likelihood of NEC prominence. Subjects with BPD did exhibit NEC prominence to an extent that approached some of the subjects with NEHI. The finding of increased NECs in BPD has been well documented and is postulated to be caused by oxidant injury. Although the histologic findings of BPD are unlikely to be confused with NEHI, we suggest caution in diagnosing NEHI in former preterm infants.

Consistent with previous reports, our patients also had hypoxemia and prolonged pulmonary morbidity out of proportion to the histologic findings. However, compared with the original disease description, patchy inflammation, fibrosis, or both were present in the majority of our cases, albeit involving only a small proportion of airways. Although patchy bronchiolitis was a distracting histologic feature in some NEHI cases, there was no difference in NEC number or distribution between patients with NEHI with or without injury at biopsy or based on history of clinical confounders. Our findings suggest that the intercurrent viral infection was a superimposed problem and not related to disease pathogenesis in these cases. We conclude that a minor degree of airway injury commonly complicates biopsies in patients with NEHI and should not preclude the clinical-radiologic-pathologic diagnosis.

The stimulus for NEC prominence in NEHI is unknown. Our immunohistochemical analysis demonstrated no active proliferation of NECs, even in NEHI subjects with viral infection detected at the time of lung biopsy. Although mitotically active NECs have been seen shortly after naphthalene-induced airway injury in animal models, they have not been noted in other studies during lung development or longer durations of airway injury, suggesting that there might be a limited window of NEC proliferative activity. Of interest, familial cases of NEHI have been reported. We speculate that NEHI is a heritable developmental aberration and not a direct response to injury. NEHI also may represent part of a disease spectrum that...
includes chronic bronchiolitis or possibly the adult lung disorder of diffuse idiopathic pulmonary NEC hyperplasia.[33]

The mechanisms underlying hypoxemia and small airway obstruction in NEHI are not known. NECs are airway chemoreceptors and release potent proinflammatory, vasoactive, and bronchoconstrictive factors in response to hypoxia and other stimuli.[34] Factors such as serotonin, bombesin, and calcitonin gene-related peptide may result in reversible airway obstruction,[35] chemotaxis of mesenchymal and inflammatory cells,[36][37] and pulmonary arterial vasodilatation.[38] Patients with NEHI have profound physiologic small airway obstruction and air trapping despite histologically patent airways. The prominent increase in NECs in respiratory bronchioles and the correlation between extent of NEC prominence and severity of small airway obstruction on infant PFTs suggest that NECs may play a causal role in the pathophysiology of NEHI. Because PFT findings in infants with NEHI were not systematically compared with those in other pulmonary disorders in this study, future investigations are necessary to determine their specificity.

Clinical practice patterns are shifting to defer diagnostic biopsy in patients meeting "typical" clinical and radiographic criteria for NEHI. [4] In this context, future studies of lung histology are likely to be limited in number and associated with greater ascertainment bias because disproportionately, the atypical cases will undergo biopsy. We observed extensive inter- and intrasubject variability in NEC number among typical NEHI cases, a finding that suggests that pathologic confirmation of the diagnosis may not always be reliable, particularly if limited airway sampling is performed. Based on this study and data reported by Brody et al,[4] we propose that lung biopsy is not needed in patients who show typical clinical and radiographic features. If performed, lung biopsy specimens should be obtained from more than one site. If concurrent airway injury is present, our data indicate that NECs should be prominent in the uninjured airways. The quantitative approaches of morphometry and cell counting that were used in this study are not routinely available and are impractical for clinical application. Until a molecular or genetic basis for NEHI is identified, we propose that the diagnosis is best established by consideration of the aggregate of clinical, radiographic, and histologic findings.

Children with NEHI experience significant pulmonary morbidity, frequent difficulties with weight gain, and extended need for supplemental oxygen (years). Despite this, all the patients with NEHI in this cohort have gradually improved over time. Multicenter longitudinal studies will better define the natural history of this disorder. Understanding the pathophysiology of NEHI and discovery of the molecular basis are needed to develop effective therapies.

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Author contributions: Dr Young: contributed to the design and conduct of the study and writing of the manuscript.

Dr Brody: contributed to the analysis of the chest CT scans and preparation of the manuscript.

Dr Inge: contributed critical patient samples and data and to the preparation of the manuscript.

Dr Acton: contributed to the performance of infant PFT and preparation of the manuscript.

Dr Bokulic: contributed critical patient samples and data and to the preparation of the manuscript.

Dr Langston: contributed to the review of the lung biopsy specimens and preparation of the manuscript.

Dr Deutsch: contributed to the design and conduct of the study and writing of the manuscript.

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