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PEDIATRIC INFECTIOUS DISEASES (I. BROOK, SECTION EDITOR)

Biofilm Formations in Pediatric Respiratory Tract Infection

Part 1: Biofilm Structure, Role of Innate Immunity in Protection Against and Response to Biofilm, Methods of Biofilm Detection, Pediatric Respiratory Tract Diseases Associated with Mucosal Biofilm Formation

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Abstract

Purpose of Review Biofilm represents an organized structure of microorganisms within an extracellular matrix attached to a surface. While the importance of biofilm in prosthetic heart valve and catheter-related infections has been known since the 1980s, the role of mucosal biofilm in human disease pathogenesis has only recently been elucidated. It is now clear that mucosal biofilm is present in both healthy and pathologic states. The purpose of this review is to examine the role of mucosal biofilm in pediatric respiratory infections.

Recent Findings Mucosal biofilm has been implicated in relationship to several pediatric respiratory infections, including tonsillitis, adenoiditis, otitis media with effusion, chronic rhinosinusitis, persistent endobronchial infection, and bronchiectasis. In these conditions, core pathogens are detected in the biofilm, biofilm organisms are often detected by molecular techniques when conventional cultures are negative, and biofilm presence is more extensive in relation to disease than in healthy tissues. In chronic rhinosinusitis, the presence of polymicrobial biofilm is also a predictor of poorer outcome following sinus surgery. Biofilm in the tonsillar and adenoidal compartments plays a distinct role in contributing to disease in the middle ear and sinuses.

Summary Key observations regarding the relevance of biofilm to pediatric respiratory infections include (1) the association between the presence of biofilm and persistent/recurrent and more severe disease in these tissues despite antibiotic treatment, (2) linkage between biofilm core pathogens and acute infections, and (3) interrelationship between biofilm presence in one tissue and persistent or recurrent infection in an adjacent tissue. A greater understanding of the significance of mucosal biofilm will undoubtedly emerge with the development of effective means of eradicating mucosal biofilm.

Keywords Mucosal biofilm · Pediatric respiratory · Respiratory tract infection · Antibiotic resistance · Extracellular · Dysbiosis

Introduction

Parsek and Singh [1] proposed criteria for establishing a "biofilm infection." These emphasize the salient morphologic features of biofilm, its role confined to a particular location and its association with infection that is "difficult or impossible to eradicate with antibiotics despite the fact that the responsible organisms are susceptible to killing in the planktonic state." Ramakrishnan et al. [2] expanded on these to emphasize that localized bacteria in biofilm may be missed in conventional blood sample or aspirates and that biofilm macrocolonies exist "in discrete areas in host tissue associated with host inflammatory cells." However, it is now clear that on mucosal surfaces, biofilm is not necessarily pathologic and that at these sites, a "healthy" versus "pathologic" microbiome must be defined. The concept of pathologic mucosal biofilm can be viewed as an extension of the concept of "dysbiosis" of the healthy microbial community in these sites. The tonsillar and adenoidal compartments likely predispose to pathologic biofilm formation due to cryptic tissue structure, lower than physiologic body temperature, and direct, repeated exposure to

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respiratory bacterial pathogens. In contrast, the paranasal sinuses, middle ear, and lower respiratory tract compartments, although not bacterially sterile, are nonetheless normally uninfected. In these areas, obstruction of ostia, impairment in mucociliary clearance, or an impairment of local innate immunity results in an environment permissive for biofilm formation. Once formed, pathologic biofilm contributes to a stable "unhealthy microbiome" that promotes disease persistence in one of two ways, namely by serving as a repository for bacterial pathogens that can egress the biofilm in the planktonic phase to cause locally recurrent infection or by disrupting the normal healthy tissue microbiome in these sites thereby promoting inflammation even in the absence of overt infection.

The physiologic processes that govern biofilm formation are genetically programmed and dependent on growth conditions, temperature, nutrient supply, and host factors. Certain defects in local innate immunity, such as a loss of function in a bitter taste receptor, a deficiency in lactoferrin or a deficiency in certain antimicrobial peptides, are associated with chronic rhinosinusitis and biofilm formation. There are distinct pathways of biofilm formation in each of the most common respiratory pathogens, and interrupting these pathways offers promising strategies to prevent biofilm formation or to eradicate established biofilm.

Biofilm Structure and Physiology

Biofilm formation is an important survival mechanism for microorganisms through attachment to surfaces [3]. Formation of biofilm is a complex process controlled by different genetic pathways depending upon growth conditions and exposure to membrane-targeting antibiotics [4]. Biofilm-associated bacteria are known to have enhanced resistance to antimicrobial agents relative to floating (planktonic) bacteria [5]. Biofilms also protect against disinfectants and host innate immune defense systems [6]. On artificial surfaces, such as urinary catheters, bacteria residing within thick adherent biofilm were shown to be up to 1000-fold more resistant to antibiotics than their planktonic counterparts [7]. It is known that in certain biofilm-related infections, an apparent response to a course of antibiotics is followed, sometimes weeks to months later, by a reemergence of infection [8]. This same process has been implicated on mucosal surfaces in several pediatric respiratory tract diseases, including adenoiditis, chronic otitis media, chronic rhinosinusitis, persistent bacterial bronchitis, and bronchiectasis.

Stages of Mucosal Biofilm Formation

Mucosal biofilm formation, similar to biofilm formation on artificial surfaces, involves discrete stages and genetically regulated processes that can theoretically be exploited for therapeutic purposes (Koo 2017). The first stage is "initial adhesion" to host epithelial cells that involves cell surface-associated adhesins and non-specific adhesive mechanisms. The second is production of "extracellular polymeric substances" (EPS) which results in more firmly adherent "irreversible" attachment of bacteria to the surface. EPS is composed of polysaccharides—sometimes referred to as the biofilm "glycocalyx" [8], proteins, nucleic acids, humic acids, and lipids [9]. EPS enhances adhesion, while forming the matrix that embeds the cells. The third stage is "early development of biofilm architecture" in which the three-dimensional structure of the biofilm forms with varying shapes, from mounds to mushroom-like microcolonies and filamentous streamers, with protective scaffolding and water channels [6, 10]. The biofilm structure is largely determined by the production of EPS [10]. The fourth stage is maturation of biofilm architecture. In this stage, there is increasing size and complexity of the biofilm architecture. The fifth stage is "dispersion of single cells from the biofilm" in which individual bacteria leave the biofilm to reenter the planktonic stage [11••]. This sometimes leaves hollow remnants of microcolonies that then become part of the water channels [10]. At any one time, the surface of biofilm may contain areas at each developmental stage [10].

Extracellular Deoxyribonucleic Acid

Extracellular deoxyribonucleic acid (eDNA) is an essential component of bacterial biofilm matrices and is required in biofilm formation and maintenance [12•]. Upon prolonged stimulation, neutrophils release decondensed chromatin and granular contents which form key constituents of neutrophil extracellular traps (NETs). This is a cell death process known as NETosis [13, 14]. Although NETosis is believed to play an important role in innate defense, such as immobilizing microbes to prevent their dispersal in the host and serving as an attachment for bactericidal enzymes including myeloperoxidase, leukocyte proteases, and cathelicidin LL-37, persistent NET exposure at sites of inflammation may contribute to persistent inflammation [14]. Hong et al. [15•] showed that non-typeable *Haemophilus influenzae* (NTHi) within middle ear chamber biofilm containing neutrophil extracellular traps were resistant to phagocytic and extracellular neutrophil killing in vitro by means of lipooligosaccharide moieties that promote biofilm formation. Several respiratory bacterial pathogens have been shown to trigger release of extracellular traps from neutrophils, including *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* [16].

The DNABII family of DNA-binding proteins or "nucleoid-associated proteins" (NAP) has been shown to be critical for the integrity of the EPS matrix of biofilms that

contain eDNA [17•]. This family, which includes integration host factor (IHF), represents a prominent group of bacterial gene regulators [18] and was found to be uniquely and critically involved in the structural integrity of the biofilms formed by NTHi [19].

The structural components and regulation of biofilm production are quite diverse amongst respiratory tract pathogens (as discussed in part II of this review) which poses additional challenges toward preventing or treating biofilm-associated diseases. It has been shown that biosurfactants produced by *Pseudomonas aeruginosa* also play important roles in biofilm formation, maturation, and dispersal [20•]. They promote microcolony formation in the initial phase and help to promote development of mushroom-shaped multicellular structures and maintain water channels between multicellular structures during biofilm maturation. Finally, they also play a role in dispersal of cells from biofilms. In contrast to these biofilm-promoting properties of bacterial biosurfactants, there are other host and bacterial biosurfactants that function to prevent the initial stage of biofilm formation (as discussed in part II of this review).

Polymicrobial Interactions in Biofilm

Many studies have observed that biofilm associated with respiratory tract diseases is most often polymicrobial, including studies of chronic rhinosinusitis [21–25, 26•], adenoiditis [27], and otitis media [22, 27]. The presence of polymicrobial, rather than single-species biofilms, has been associated with greater preoperative disease severity and poorer post-surgical outcome in studies of chronic rhinosinusitis patients [23]. The social interactions between bacteria involved in polymicrobial biofilm include exchange of genetic material and antibiotic resistance factors [28].

The Concept of Biofilm-Associated Infection

Parsek and Singh proposed the following criteria for establishing a biofilm infection: (a) The infecting bacteria are adherent to some substratum or are surface associated. (b) Direct examination of infected tissue shows bacteria living in cell clusters, or microcolonies, encased in an extracellular matrix. The matrix may often be composed of bacterial and host components. (c) The infection is generally confined to a particular location. Although dissemination may occur, it is a secondary phenomenon. (d) The infection is difficult or impossible to eradicate with antibiotics despite the fact that the responsible organisms are susceptible to killing in the planktonic state [1]. Ramakrishnan et al. [2] expanded on these to include the following: "Culture-negative result despite clinically documented high suspicion of infection (localized bacteria in biofilm may be missed in conventional blood sample or aspirate); and ineffective host clearance evidenced by location of bacterial cell clusters (macrocolonies) in discrete areas in host tissue associated with host inflammatory cells."

Mucosal Biofilm in Otherwise Healthy Subjects

Mladina et al. [29] performed a blinded study examining sinus tissue for the presence of mucosal biofilm in patients without chronic rhinosinusitis (CRS). The samples included healthy sphenoid sinus tissue from 48 patients undergoing pituitary gland surgery and ethmoidal sinus mucosa from 17 patients undergoing endonasal endoscopic orbital decompression (EEOD) for Graves' disease. The tissue samples were submitted blindly along with similar samples from patients with CRS to two scanning electron microscope experts. All samples were examined by SEM. The authors found that biofilm was present in 45 of 48 pituitary gland surgery patients (94%) and in all 17 EEOD patients (100%) [29].

Abnormalities of the Nose and Sinus Microbial Community ("Microbiome") in Chronic Rhinosinusitis

The evolving view of bacterial involvement in CRS has expanded beyond that of infection with individual pathogens to considerations of increased bacterial burden, biofilm formation, and alterations in the microbial community ("microbiome"). The "microbiome" is the universe of culturable and non-culturable microorganisms present in a specific ecological niche, such as the gastrointestinal tract or the sinus mucosa. Analysis of the microbiome has gained great interest as a means of studying host-microbial interactions in diseases, including those of the respiratory tract and sinuses. These analyses employ broad platform molecular techniques, including microarrays and sequencing methods allowing for identification of the full microbiome based on microbial RNA. The most commonly used technique is "pyrosequencing" which identifies bacterial species based on the conserved 16S eubacterial ribosomal gene. Important characteristics of the microbial community include bacterial diversity (richness and evenness), over- or underrepresentation of specific bacterial species, and bacterial "load" or "burden."

Studies of the CRS microbiome in healthy versus diseased adult sinus tissue have reported that the "burden" of bacterial organisms in the sinus mucosa is not significantly altered in CRS, but the bacterial diversity (richness, evenness) is reduced, and the burden of certain bacteria, most notably *Staphylococcus aureus* is increased (reviewed in [30]). Abreu et al. observed that in comparison with the "normal healthy" sinus microbiome, the CRS microbiome is characterized by an overrepresentation of certain "harmful" bacterial taxa or species (such as *Corynebacterium tuberculostearicum*) and underrepresentation of other "protective" bacteria, most

notably *Lactobacilli* (e.g., *Lactobacillus sakei*) [31•]. Studies such as this also provide some hope that strategies to correct the dysbiosis in CRS may lead to improved sinus health.

Role of Innate Immunity in Protection Against and Response to Biofilm

Nitric Oxide

Nitric oxide (NO) has antimicrobial effects in the upper and lower airway. This function is partly attributable to stimulation of increased ciliary beat frequency but also relates to direct antimicrobial effects due to complex reactivities between NO radical superoxide, metals, and thiols [32]. NO is produced "constitutively" in high concentration in the nasal and sinus epithelium (parts per million concentrations) in stark contrast to its very low constitutive production in the lower airway (parts per billion concentration). In the nasal and sinus epithelium, NO is also induced by innate signaling through bitter taste receptors which are activated by quorum-sensing molecules from bacteria [33]. The extent to which quorum-sensing molecules from bacteria account for the "constitutive" production of NO in the upper airway is presently unknown. The concentration of NO in the upper airway is sufficient to exert antimicrobial effects [34]. The best example of this is the demonstration that NO production in sinus airway epithelial cells grown at air-liquid interface culture is sufficient to prevent the growth of *Pseudomonas aeruginosa* (see discussion of bitter taste receptors below) [35].

The syndrome of primary ciliary dyskinesia (PCD) is associated with a dramatic reduction in nasal NO; however, the extent to which this reduction in NO is a cause or consequence of sinusitis in these patients is unclear [34]. Indeed, NO production by PCD primary epithelial cells grown at air-liquid interface was found to be similar to that in non-PCD epithelial cells [36]. However, nasal NO levels are reduced in the presence of sinus infection [34] and, in addition, reduced nasal NO levels in PCD patients have been shown to correlate with sinus aplasia/hypoplasia of the sinuses [37]. Nasal NO levels are also lower in cystic fibrosis (CF) patients whereas lower airway (alveolar) NO levels are no different compared with healthy controls [38], again likely a reflection of ongoing sinusitis rather than a primary NO production defect in CF. Nasal NO measurement is a sensitive and specific test for PCD in the setting of a high clinical suspicion for PCD and when CF has been ruled out [39].

Innate Signaling Through Bitter Taste Receptors

Bitter taste receptors are a family of G protein-coupled receptors that signal by inducing a transient intracellular calcium flux and increasing ciliary beat frequency. Activation of the receptor induces production of NO in sinus epithelial cells. Lee et al. discovered that one of the bitter taste receptors, T2R38, is activated by a quorum-sensing molecule from *Pseudomonas aeruginosa* associated with biofilm formation [35]. A common bitter taste receptor polymorphism (TAS2R38 variant) is associated with reduced signaling, reduced NO production, reduced CBF, and increased growth of *P. aeruginosa* in air-liquid cultures of human airway epithelial cells. The effect of TAS2R38 on killing of *P. aeruginosa* is dependent on NO. In CRS patients, the TAS2R38 genotype correlated with the presence of sinonasal Gram-negative infection suggesting a mechanistic link between a deficiency in innate signaling and increased bacterial infection [35].

The TAS2R38 gene polymorphism affects taste perception as reflected by a lack of gustatory sensitivity to the bitter compound phenylthiocarbamide (PTC) [40]. A strong inverse correlation was shown between PTC taste sensitivity and the ability of sinonasal swab bacteria to form biofilm in vitro in patients with non-polypoid CRS [40]. Three different bitter taste receptors, namely T2R38, T2R4, and T2R16, have been found expressed throughout the sinonasal cavity [33].

Innate Antimicrobial Peptides

There are numerous innate antimicrobial peptides secreted in airway secretions. Several of these are produced constitutively in respiratory secretions, but their production can also be induced through pathogen-sensing receptors, including Toll-like receptors and bitter taste receptors. Certain of the antimicrobial peptides have been shown to play a role in inhibiting biofilm formation.

Lactoferrin

Lactoferrin is an iron-binding cationic glycoprotein secreted by airway exocrine glands and neutrophils. It is (after lysozyme) the second most abundant antimicrobial peptide in respiratory secretions [41]. An early study reported that lactoferrin blocks biofilm formation in vitro [42]. This was shown to occur at lactoferrin concentrations (100 mcg/ml) below those necessary to kill or prevent bacterial growth and well below the level present in airway secretions (0.4–1.0 mg/ml). By chelating iron, lactoferrin was found to stimulate twitching, a specialized form of surface motility [43], causing the bacteria to wander across the surface instead of forming cell clusters and biofilms.

SPLUNC1/BPIFA1

SPLUNC1/BPIFA1 (hereafter referred to as SPLUNC1) has been shown to inhibit the biofilm-forming capacity of *Burholderia cepacia* in vitro [44•]. This property of SPLUNC1 was shown to reside in the α4 helix, and

neutrophil elastase which cleaves SPLUNC1 was shown to enhance SPLUNC1's antimicrobial effects [44•]. A SPLUNC1 deficient mouse (SPLUNC1-/- knockout mouse) showed an increased susceptibility to *Klebsiella pneumoniae* lung infection [45•]. SPLUNC1 was further shown to reduce airway surface tension thereby exerting an important biosurfactant property. SPLUNC1 was also shown to inhibit both *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* biofilm formation in vitro [45•, 46•, 47•]. Other biosurfactants derived from bacteria have been described that inhibit biofilm formation [20•, 48, 49].

In addition to its antimicrobial and biosurfactant properties, SPLUNC1 protein has been shown to modulate airway surface liquid (ASL) homeostasis by acting as an endogenous inhibitor of the epithelial Na(+) channel (ENaC). This has special relevance to cystic fibrosis (CF) airway disease. Tarran and Redinbo proposed that SPLUNC1 serves to plug the operational ENaC "drain" that removes sodium ions and water from the ASL overlying airway epithelia [50•]. At normal pH in healthy lungs, SPLUNC1 can effectively contact ENaC and prevent the proteolytic activation of this channel. However, in CF, the decrease in ASL pH relative to healthy ASL is sufficient to disrupt SPLUNC1's contact with ENaC, thereby allowing ENaC to remove Na^+ ions and water and causing ASL dehydration and mucostasis [51]. Levels of SPLUNC1 are actually increased by immunostaining in CF in diseased small airways compared with similar sized airways in non-CF lungs where staining is absent [52], whereas SPLUNC1 is incapable of functioning normally in CF. In support of this, it was shown that experimentally elevating the CF ASL pH prevents cleavage of ENaC and restores ASL volume homeostasis [51]. The complete SPLUNC1 molecule appears essential in pH-dependent ENaC control [50].

Tsao et al. examined a large group of adult CRS patients with and without *Pseudomonas aeruginosa* infection and found that the level of SPLUNC1 mRNA expression and protein immunohistochemical staining was reduced in the patients with *P. aeruginosa* infection [53•]. SPLUNC1 was expressed primarily in submucosal glands. Patients infected with *P. aeruginosa* also had a significantly higher need for repeated sinus surgery [53•].

Other Antimicrobial Peptides

Benicasa et al. found that polysaccharides (components of biofilm EPS, such as alginate and cepacian) produced by *P. aeruginosa*, *Klebsiella pneumoniae*, and *Burkholderia cepacia* complex inhibited the antibacterial activity of the antimicrobial peptides cathelicidin LL-37 and beta-defensin hBD-3 [54]. This inhibitory activity is not simply due to ionic interaction between the negatively charged polysaccharides and the cationic antimicrobial peptides but also involves other structural features of the interactions.

Extracellular DNA (eDNA), another component of the biofilm matrix, was shown to inhibit the function of human β-defensin-3 (hBD-3), including the ability of hBD-3 to inhibit the formation of *Haemophilus influenzae* biofilm in vitro [55•].

Impairment of Leukocyte Killing in Biofilms

Leid et al. [56] found that human neutrophils adhere to and penetrate *Staphylococcus aureus* biofilm under "physiologic" conditions of shear stress in vitro; however, the neutrophils are incapable of ingesting and killing the biofilm bacteria. The authors also showed that biofilms are more akin to an extremely porous hydrogel than a solid, rigid structure [57]. Other mechanisms of impairment of neutrophil killing have been described in biofilms, such as the ability of alginate to protect *P. aeruginosa* in biofilms from phagocytosis by IFN-gamma-activated macrophages [58•] (reviewed in [59]).

Figure 1 summarizes important features of mucosal biofilm structure and composition and elements of normal host innate immunity that protect against pathologic biofilm.

Methods of Biofilm Detection on Tissues

Scanning Electron Microscopy, Transmission EM, and Confocal Scanning Laser Microscopy

Multiple techniques for biofilm detection have been described. Scanning electron microscopy (SEM) and transmission EM (TEM) have the advantage of high magnification and are the only techniques that provide ultrastructural confirmation of biofilm presence. Technical artifacts may arise due to sample dehydration and distortion of the surface. Disadvantages of SEM include small sample size potentially introducing sampling artifacts. Confocal scanning laser microscopy (CSLM) has the advantage that specimens can be imaged without fixation or dehydration, and specific bacteria or fungi can be stained with fluorescent markers. The FISH assay utilizes either universal bacterial probes, such as EUB338 [60] or species-specific primers based on unique sequences in the 16S ribosomal RNA gene. The LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen Corp., Carlsbad, CA) employs two nucleic acid stains—green-fluorescent SYTO® 9 stain that labels both live and dead bacteria and red-fluorescent propidium iodide that penetrates only bacteria with damaged membranes. CSLM is used to confirm the presence of biofilm on the mucosal surface. No bacterial-specific probes are employed.

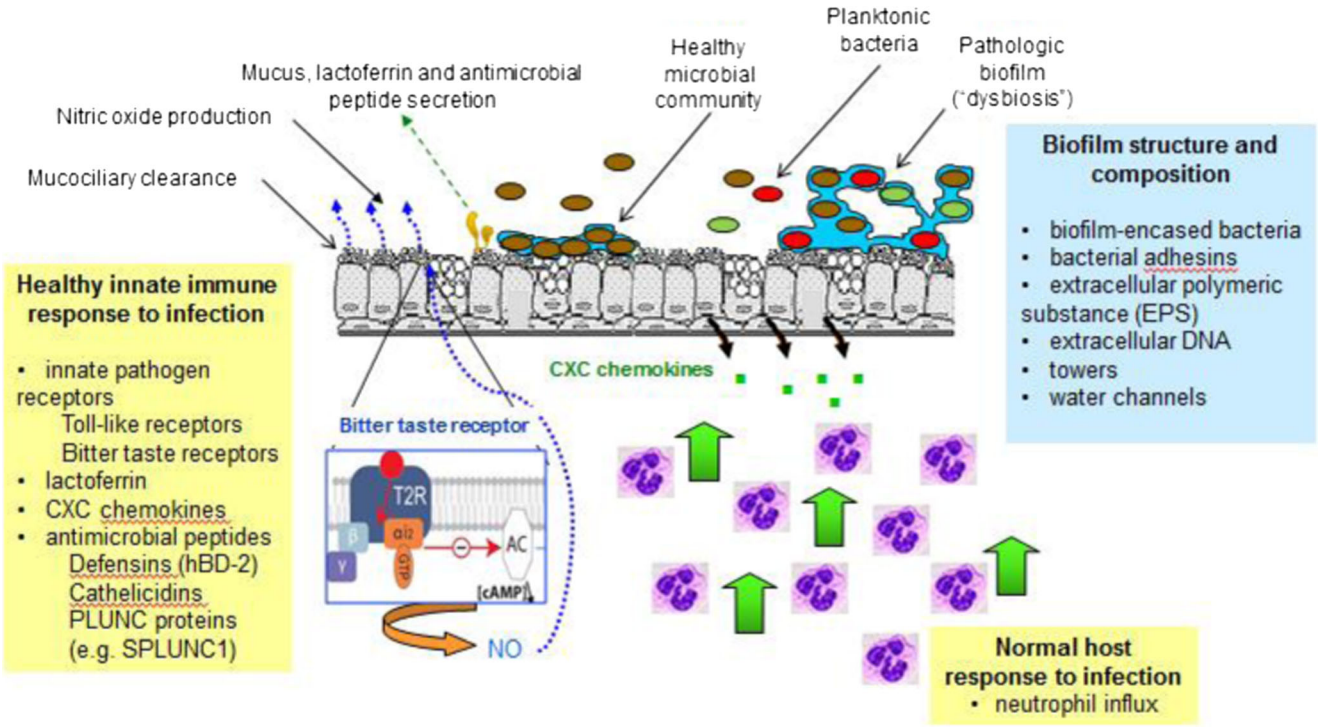

Fig. 1 Important features of mucosal biofilm structure and composition and elements of normal host innate immunity that protect against pathologic biofilm

A direct comparison of techniques in a sheep model of rhinosinusitis concluded that CSLM is more objective for documenting biofilm presence than SEM or TEM owing to inherent flaws, sampling error, and subjectivity involved in SEM and TEM [61]. Another study determined that the BacLight method was more specific than CSLM/FISH for biofilm detection in sinus tissues [62, 63]. A concern is whether any techniques other than SEM or TEM are sufficiently rigorous to define "biofilm," since they do not provide ultrastructural confirmation of the presence of biofilm.

Biofilm-Forming Capacity In Vitro

The Calgary Biofilm Detection Assay is an in vitro test used to assess the ability of a bacterial isolate to form biofilm. The bacteria are cultured on a 96-well plate with round pegs positioned over the plate. A semiquantitative analysis utilizing uptake of crystal violet in the biofilm has been developed [64]. The LIVE/DEAD® BacLight™ Bacterial Viability Kit (see above) can also be used to assess biofilm formation in vitro on 98-well chamber slides [65]. Prince et al. [21] used the Calgary Biofilm Detection Assay to examine 159 CRS patients with mucopurulence and found that 28.6% of patients had biofilm-forming capacity. Polymicrobial cultures, *Pseudomonas aeruginosa*, and/or *Staphylococcus aureus* comprised 71% of samples. Post-surgical cases had a higher prevalence of biofilm-forming capacity than surgery-naïve patients (30.7% versus 15%).

Intracellular Bacteria in CRS

Intracellular (intraepithelial) *Staphylococcus aureus* (IESA) was first reported in sinus epithelium by Clement et al. [66] using both confocal and transmission electron microscopy imaging and subsequently reported by Plouin-Gaudon [67]. In the latter study, 11 of the 17 patients in whom IESA was demonstrated relapsed for rhinosinusitis within the 12 months following endoscopic sinus surgery. In a study by Corriveau et al. [68] using a peptide nucleic acid-fluorescence in situ hybridization (FISH) assay, IESA were found in the epithelium of CRS patients but were also seen in some healthy controls raising question of the pathologic significance. In a study using similar *S. aureus* fluorescence in situ hybridization probe with propridium iodide counterstain and confocal scanning laser microscopy (CSLM), IESA were detected in 56% of CRS patients undergoing sinus surgery but none of 8 healthy controls [69]. Simultaneous analysis revealed the presence of *S. aureus* biofilm in 100% of the IESA-positive tissues and in 50% of the IESA-negative CRS patients. In another study from this group, 51 patients with refractory CRS were evaluated both for the presence of IESA as well as mucosal biofilm [70]. The authors found that 20 patients (39%) were positive for both IESA and biofilm, 16 patients (31%) had biofilm alone, and 15 patients (29%) had no evidence of *S. aureus*. Patients with IESA had a significantly higher risk of late clinical and microbiological CRS relapses, whereas patients with biofilm alone without coexisting IESA did not have a worse

prognosis [70]. Given that there are fewer studies of IESA than of biofilm in CRS, the significance of IESA on CRS pathogenesis remains unclear. The contribution of IESA to persistent mucosal inflammation in CRS may not become clear until strategies to selectively eradicate mucosal biofilm are developed (see below).

Pediatric Respiratory Tract Diseases Associated with Mucosal Biofilm Formation

Tonsillitis

Tonsillar biofilm was first described by Chole and Faddis [71] within the tissue and crypts of 19 inflamed tonsils using a light and transmission electron microscopy. A comparison was made to 4 tonsils removed because of hypertrophy and obstruction. Features consistent with biofilm were found in the crypts of 11 of 15 infected tonsils. However, small clusters of bacterial colonies were seen in 3 of 4 tonsils removed because of hypertrophy. Galli et al. [72] studied tonsillar tissue from 8 patients (mostly adults) with recurrent infection despite repeated treatment with anti-inflammatory agents and antibiotics. Tissues were cultured using conventional methods and examined by scanning electron microscopy for detection of biofilm. Conventional bacterial cultures yielded bacteria in 62.5% of tissues, including *Staphylococcus aureus* in 3 and alpha-hemolytic *Streptococcus* in 5 specimens. Biofilm was also detected in 62.5% of the specimens. It is noteworthy that in this same study, 8 adenoid tissues from other patients were studied and grew *Haemophilus influenzae* in 6, *Streptococcus pyogenes* in 1, and alpha-hemolytic *Streptococcus* in 1 patient.

Adenoiditis

Two studies by Galli et al. [73, 74] found that *Haemophilus influenzae* was the most common pathogen cultured from adenoidal tissue from pediatric patients with recurrent adenotonsillitis in association with biofilm formation.

In a study of otitis-prone children (i.e., children with recurrent acute otitis media), Hoa et al. [27] performed adenoidectomies and examined the adenoids for the presence of biofilm. They found that all of the otitis-prone children had biofilm encompassing > 85% of the adenoidal surface area by SEM. In contrast, FISH accompanied by CSLM imaging demonstrated patchy adenoidal biofilms. Each of the adenoidal biofilms contained one or more otitis media pathogens, including *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* [27].

A study by Torretta et al. [75] examined 135 adenoid biopsies from the nasopharyngeal dome (ND) and compared them with adenoidal tissue near the opening of the eustachian tube (ET) in a group of 45 children with chronic or recurrent middle ear infections. They found that biofilm-producing bacteria were detected more frequently in the ET samples compared with the ND samples. This study supports the postulated role of biofilm seeding of the eustachian tube in the pathogenesis of chronic or recurrent middle ear infections.

Enlarged Adenoidal Pads (Adenoiditis) Harboring Bacteria That Cause CRS

Biofilm in the nasopharynx of children with CRS may act as a reservoir for bacterial pathogens resistant to standard antibiotics. Coticchia et al. [76•] studied the prevalence and extent of adenoidal biofilm in 7 pediatric CRS versus 9 obstructive sleep apnea (OSA) cases using ultrastructural SEM analysis of biofilm. They found that the mean surface biofilm area in adenoidal tissue was 94.9% of the pediatric CRS patients versus 1.9% of the pediatric OSA cases. Other studies showed a correlation between the presence of rhinosinusitis and infected adenoid core pathogens [77, 78]. These studies provide a rationale for adenoidectomy as a first step in pediatric CRS surgical management.

Chronic (Persistent) Otitis Media with Effusion

Hall-Stoodley et al. [79] used confocal scanning laser microscopy (CSLM) to examine middle ear mucosa (MEM) biopsies from 26 children who underwent tympanostomy tube placement for otitis media with effusion (50%), recurrent otitis media (77%), or both diagnoses (27%). Control uninfected MEM was also obtained from 3 children and 5 adults undergoing cochlear implantation. Using generic stains and species-specific probes, they identified the presence of biofilm on 92% of 50 MEM specimens from children with OME and recurrent OM and in none of the 8 control MEM specimens [79]. Interestingly, 24 of 24 middle ear effusions were PCR-positive for at least 1 otitis media pathogen, whereas only 6 of 27 (22%) effusions were culture positive for any pathogen.

In this study, the authors also found that at least in some patients with recurrent OM, bacterial biofilms were present during periods of clinical remission. This finding suggested that in some cases, consecutive episodes of acute OM could be driven by organisms in the biofilm [80], although the frequency with which this accounts for recurrent OM is unclear.

Otitis Media with Effusion

Similarly, metabolically active bacteria have been detected in culture-negative pediatric middle ear effusions. Otitis media with effusion (OME) is characterized by the presence of fluid in the middle ear cavity, behind an intact tympanic membrane [81]. It is considered a benign condition and was formerly believed to be a sterile condition. However, studies utilizing molecular techniques have

identified the presence of pathogenic bacteria in a high percentage of cases. Van Hoecke et al. [81] examined middle ear fluid and adenoid tissue from 21 children with chronic otitis media with effusion chronic (persistent) otitis media with effusion (COME) by performing cultures and examining middle ear fluid and adenoid tissue for bacterial clusters using fluorescence in situ hybridization (FISH) and CSLM. The middle ear effusions (MEE) were 64.7% culture positive for bacteria and 47.0% culture positive for *H. influenzae*, *M. catarrhalis*, *S. aureus*, and/or *S. pneumoniae*. All 21 adenoid samples were culture positive for one or more of these pathogens. The same bacterial species were found in MEE and adenoid for 84.6% of the patients, and in 81.2% of these, the bacteria in the two sites were of the same bacterial genotype. FISH and CSLM demonstrated the presence of *H. influenzae*-specific biofilm structures in 5 of the 8 culture-positive MEEs that were tested, but in neither of 2 culture-negative MEEs. The authors concluded that the adenoid acts as a reservoir for bacteria in MEE and confirmed that biofilms (consisting of *H. influenzae* in at least half of the cases) were present in the MEE of children with COME.

Chronic Rhinosinusitis

Biofilm formation on sinonasal mucosal surfaces was first described in 2004 [82] and later in several other studies [83–88]. Virtually, all of these studies involved examining sinus tissue obtained at the time of surgery for the presence of biofilm. In one study, the presence of biofilm was associated with more severe preoperative disease based on radiologic and nasal endoscopic scoring and worse sinus symptom and nasal endoscopy scores 16 months after surgery [85]. Single organism *Haemophilus influenzae* biofilm was associated with mild clinical and radiographic disease and normalization of sinus mucosa a short time following surgery. In contrast, polymicrobial biofilm or biofilm containing *Staphylococcus aureus* was associated with more severe disease and a poorer postoperative course [23]. The presence of bacterial biofilm was also strongly associated with persistent mucosal inflammation after endoscopic sinus surgery (ESS) [86]. One study showed that the likelihood of detecting bacteria with biofilm-forming capacity increases in relation to prior ESS possibly reflecting the severity of their disease [21]. Similarly, Zhang et al. found an association of biofilm-forming capacity in vitro from clinical samples with prior sinus surgeries and nasal steroid use in the month prior to sample collection but no association with the presence of nasal polyps, allergy, or Samter's triad [25] at least suggesting that biofilm may be of greater relevance in non-polypoid CRS. In another study, Bendouah et al. [89] found that the biofilm-forming capacity of bacteria (specifically *Pseudomonas aeruginosa* and *Staphylococcus aureus*) isolated from CRS patients at the time of surgery was a predictor of poor evolution of CRS symptoms and endoscopic severity in patients followed at least 1 year post-ESS. A similar relationship was not found for biofilm-forming capacity of coagulase-negative *Staphylococcus* in these same patients. In total, these studies suggest that mucosal biofilm is a marker of more severe mucosal disease and a predictor of poorer outcome following sinus surgery.

Most studies of CRS have not subcategorized patients as CRS without or CRS with nasal polyps. One study [90] found biofilm to be present in only 2 of 12 patients with nasal polyps, a lower prevalence than reported in most other studies of CRS.

When the studies employing SEM, TEM, or CSLM are taken in total (excluding the study confined to patients with NP), the prevalence of biofilm in the CRS case series cited above was 56.3%. A limitation of the above studies of CRS is that they were done at the time of sinus surgery when the sinus tissues were more likely to be inflamed. Although they yield important insights about the contribution of biofilm to the persistence of disease, they do not address the common clinical scenarios in which patients who have had adequate sinus ventilation by surgery have persistent sinus inflammation following surgery even in the absence of overt infection. Potential explanations for persistent inflammation in this setting include poor mucociliary clearance (which is most clearly present in the setting of cystic fibrosis or primary ciliary dyskinesia), persistent exposure to irritants or pathogenic viruses or bacteria (e.g., in the setting of repeated viral exposures), persistent allergen exposure, or persistent inflammation driven by dysbiosis. It is in regard to dysbiosis that the presence of pathologic biofilm contributes to persistent disease. Cope and Lynch put forth the hypothesis that CRS represents a state of dysbiosis by contrasting the healthy sinus microbiome with the unhealthy disease-associated microbiome. Although dysbiosis is not considered synonymous with biofilm, nearly all publications on mucosal biofilm have characterized biofilm as a pathologic state. However, there are a few studies that have described a healthy microbial biofilm, for instance, in the gut [91]. Furthermore, the study by Dickson et al. [92] analyzed the microbiome of bronchoalveolar lavage (BAL) fluid with and without its cellular composition and found that the removal of host cells from BAL fluid decreased the total bacterial content and altered the community composition, implying that specific bacterial community members are cell-associated. It seems therefore important to conceptualize a "healthy biofilm" in the context of the healthy microbiome and "pathologic biofilm" in the context of dysbiosis (disease-associated microbiome). In support of this, it has been demonstrated that healthy sinus mucosa is not sterile but rather a stable microbial community comprised of diverse microorganisms. Cope and Lynch [93] hypothesized that the microbial community "interacts with the host epithelium to maintain

immune homeostasis" that is, "resistant to colonization by or outgrowth of harmful sinus pathogens." In contrast, perturbations in the healthy sinus microbiome can lead to the evolution of a "disease stable community" characterized by reduced microbial diversity that favors inflammation-tolerant pathobionts [93]. Pathologic biofilm is therefore an important feature of dysbiosis. Furthermore, Cope and Lynch's hypothesis implies that persistent sinus inflammation can result from dysbiosis in the absence of overt infection and is a reasonable corollary to the criteria for biofilm infection put forth by Parsek and Singh [1]. The importance of dysbiosis as a driver of CRS pathogenesis is deserving of greater attention and experimental validation. To date, there have been no studies comparing the microbiome and biofilm features of CRS patients following endoscopic sinus surgery comparing those with persistent inflammation to those whose sinuses appear healthy. The same could be said about examining the importance of pathologic biofilm in other disease processes, such as chronic wet cough and persistent bacterial bronchitis.

Jardeleza et al. [94] compared sinonasal tissue samples from CRS patients with and without nasal polyps and compared them with control tissues. In each tissue, the presence of *S. aureus* biofilm was determined using FISH and in parallel the tissue was analyzed using a human inflammasome PCR array. The biofilm-positive CRS tissues showed the greatest number of differentially expressed genes compared with controls. In contrast, no difference was found between the biofilm-negative CRS tissues versus controls. This study suggests that the presence of *S. aureus* biofilm in CRS tissues activates an intracellular inflammasome response that could, at least potentially, contribute to disease persistence or early relapse. This is consistent with the notion that dysbiosis of the normal sinus microbiome may be sufficient to drive persistent inflammation. Another study by this same group found that in 34 CRS patients who underwent ESS and received multiple courses of culture-directed antibiotics following surgery that in 79% of cases there was persistence of the same *S. aureus* strain (determined by pulsed-field gel electrophoresis) in their paranasal sinuses following ESS and antibiotic treatment [95]. This study suggests that relapses of CRS following appropriate culture-directed antibiotic treatment may be partly explained by the formation of biofilms in the paranasal sinus tissues.

In contrast to the prognostic significance of polymicrobial sinonasal biofilm in CRS, a study examining postoperative sinus tissues for the presence of intracellular (intraepithelial) *S. aureus* (IESA) found that IESA was more prevalent in CRS without nasal polyps (CRSsNP) than in CRS with nasal polyps (CRSwNP) or controls (80% vs 56% vs 38%, respectively), but the presence of IESA was unassociated with symptom or endoscopic severity scores at the time of surgery nor 12 months postoperatively [96].

Interrelationship Between Respiratory Pathogens in the Adenoids, Sinuses, and Middle Ear

Studies linking biofilm in the adenoids of children with CRS and a correlation between the presence of rhinosinusitis and infected adenoid core pathogens were discussed above [76•, 77, 78]. A study by Brook, Yocum, and Shah [97] found an association between bacterial pathogens in the middle ear and in the sinuses in children with chronic otitis media with effusion (OME) and (CRS). Cultures were obtained from 32 children with concurrent chronic otitis media with effusion and maxillary sinusitis who underwent tympanostomy tube placement. The most common organisms isolated were *H. influenzae*, *Streptococcus pneumoniae*, *Prevotella* species, and *Pepotostreptococcus* species, with a microbiological concordance between the ear and sinus cultures of 69% in culture-positive patients. A study by Davcheva et al. [98] followed on this observation and examined cultures from the adenoid tissue in children with concurrent chronic OME and CRS. In this study, 20 children with chronic OME undergoing adenoidectomy were studied and 9 of them had concurrent CRS. The following bacterial pathogens were isolated from the adenoidal tissue in these cases: *H. influenzae* in 7 (35%), *S. pneumoniae* in 6 (30%), *M. catarrhalis* in 4 (20%), and *S. aureus* in 2 (10%) patients and group A *Streptococcus pyogenes* in 1 (5%). In all but one case, only one bacterium was isolated from the adenoids. This study did not examine the adenoidal tissue for biofilm, per se.

Persistent Endobronchial Infection or "Protracted Bacterial Bronchitis" and Bronchiectasis

The term "persistent endobronchial infection" or "protracted bacterial bronchitis" (PBB) has been proposed to describe a persistent endobronchial infection, particularly in children, in which a major underlying inherited risk factor, such as cystic fibrosis, PCD, or antibody deficiency, cannot be identified [99, 100]. This condition is characterized by a chronic "wet" or productive cough lasting for a minimum of 4 weeks that resolves with appropriate antibiotic treatment. It is generally associated with neutrophilic airway inflammation and bacterial infections of the conducting airways. It is more commonly reported in children living in poor communities and may be the forerunner to the development of "non-cystic fibrosis bronchiectasis" [100, 101]. Although biofilm studies of this condition are very limited, its association with persistent infection with organisms such as non-typeable *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* makes formation of mucosal biofilm in the airways likely, especially if the chronic wet cough persists or reemerges with the same organism following appropriate antibiotic treatment.

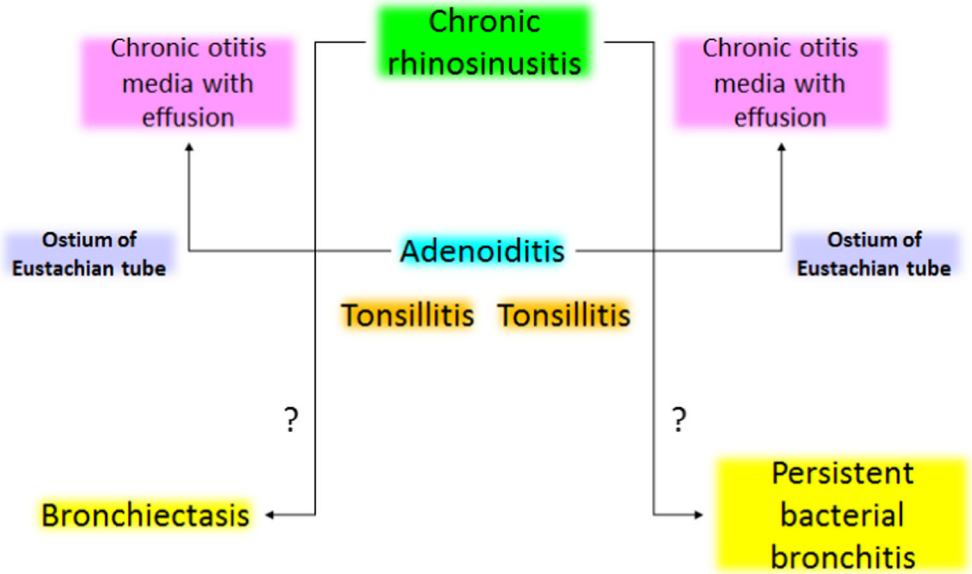

Fig. 2 Sites and interactions of biofilm in pediatric respiratory disease. A linkage has been described between biofilm-associated pathogens in the adenoids and those isolated from the ostium of the Eustachian tubes, the middle ear in chronic otitis media with effusion, and in the sinuses in chronic rhinosinusitis. The linkage between chronic rhinosinusitis and bronchiectasis and persistent bacterial bronchitis is shown with question marks, because these are presumed linkages without direct experimental proof

Figure 2 summarizes the sites and interactions of biofilm in pediatric respiratory disease.

Conclusions

Our understanding of the importance of bacterial biofilm in the pathogenesis of human disease has advanced beyond that of biofilm attachment to artificial surfaces to a growing appreciation of the importance of mucosal biofilms. In this regard, it is now also appreciated that host innate immunity plays an important role in protection against mucosal biofilm and biofilm-associated infection. An emerging concept is that mucosal biofilm is normally present in respiratory tissues and a property of the mucosal microbiome. Therefore, a distinction should be made between a "healthy" versus "pathologic" mucosal microbiome or mucosal biofilm. Pathologic mucosal biofilm can be viewed as a form of dysbiosis of the healthy microbial community in these sites. Mucosal biofilm has been described as a feature of many pediatric respiratory tract diseases, including tonsillitis, adenoiditis, chronic otitis media with effusion, otitis media with effusion, chronic rhinosinusitis, persistent endobronchial infection ("protracted bacterial bronchitis"), and bronchiectasis. However, relatively few of these descriptions have rigorously defined a "healthy biofilm" versus "pathologic biofilm" in these tissues. Nonetheless, in chronic respiratory diseases, the mucosal biofilm present is well represented with the appropriate respiratory pathogens. Furthermore, there is a strong connection between biofilm-associated pathogens in chronically inflamed adenoids in children and the same pathogens in chronic otitis media and chronic rhinosinusitis suggesting a causal link.

Another emerging concept is that biofilm-associated bacterial pathogens may promote disease persistence in one of two ways, namely by serving as a repository for bacterial pathogens that can egress the biofilm in the planktonic phase to cause locally recurrent infection or by disrupting the normal healthy tissue microbiome in these sites thereby promoting inflammation even in the absence of overt infection.

Compliance with Ethical Standards

Conflict of Interest Daniel L. Hamilos declares that he has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by Dr. Hamilos.

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- •• Of major importance

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