Significance and Innovation: Community- acquired viral and bacterial pneumonia (CAP) and asthma exacerbations due to viral pathogens are prevalent and costly reasons for hospitalizations in children. CAP alone accounts for ~1.8 million healthcare visits annually in the U.S. and is the third most prevalent reason for hospitalization in children.[1,2,3] Additionally, these respiratory diseases can occur concurrently or separately, require different treatment strategies, and importantly, etiology can be difficult to distinguish.[4,5,6] Identification of bacterial CAP is difficult because blood cultures, though low risk, rarely identify an organism (<5% of the time), while the use of bronchoalveolar lavage fluid cultures, which has a higher yield, requires high risk and invasive bronchoscopy.[7] Accurate diagnosis of bacterial respiratory infections is important because empiric antibiotic therapy confers no benefit for children with non-bacterial infections, but places children at risk for adverse drug reactions, and treatment-related complications, e.g., *Clostridium difficile* colitis, and antibiotic resistance.[8] Conversely, delaying appropriate antibiotic care can lead to poor outcomes including septic shock and death.[9] The Pediatric Infectious Diseases Society and Infectious Diseases Society of America national guideline highlights the need for better diagnostics in CAP by encouraging research on developing "diagnostic tests that are noninvasive yet sensitive and specific in documenting clinical disease caused by single pathogens or combinations of pathogens."[9,10]

Currently non-invasive biomarkers do not exist to differentiate bacterial from viral CAP reliably. Conventional biomarkers when used alone have limited ability in suggesting etiology or severity in pediatric CAP, which makes clinical management decisions challenging.[11] In a previous study by the PI, in 477 children enrolled with CAP, conventional biomarkers such as white blood count (WBC), absolute neutrophil count (ANC), C-reactive protein (CRP), and procalcitonin (PCT) all had a minimal ability (AUC < 0.60) to discriminate severe and non-severe disease.[11] It has been shown that an elevated PCT is suggestive of bacterial infection.[12] However, bacterial infection alone is not a strong predictor of severity in children with CAP. The most prevalent and prominent pathogen of severe pneumonia in children was Respiratory Syncytial Virus (RSV), not a bacteria.[13] Additionally, 52-65% of asthma exacerbations have an underlying viral pathogen with rhinovirus, RSV, and human Metapneumovirus being the most prevalent viral triggers.[14] Although different clinical pathways exist for the management of asthma exacerbations versus viral CAP, sometimes the pathophysiology of the two conditions can be challenging to ascertain causing delays in receipt of steroids for children with asthma exacerbations when misdiagnosed for viral CAP or unnecessary steroid treatment in children with viral CAP when misdiagnosed as asthma. Children with asthma have varying phenotypes and endotypes that have been previously shown to affect response to treatment and may be a point of focus for future studies using the proposed technology.[15] The development of a non-invasive and accurate diagnostic test that could rapidly differentiate community- acquired bacterial pneumonia (CAP) from isolated viral lower respiratory tract infections or asthma exacerbations triggered by a viral pathogen, would greatly improve clinical management, patient safety and health care costs.

The field of laser spectroscopy has advanced substantially in the past decade to allow for the potential of evaluating a non-invasive sample, exhaled breath, using a powerful new technology, optical frequency combs, a 2005 Nobel Prize-winning laser technology. Breath analysis is the first biomedical application of the comb. The comb, a quantum sensing technology, probes breath molecules as quantum mechanical systems. Optical frequency combs are a novel type of laser light source whose spectral output comprises hundreds of thousands of discrete, perfectly evenly spaced frequencies, analogous to the teeth on a comb. By changing the cavity length/repetition rate of the laser, the frequencies of these lines can be precisely tuned.[16] The unique properties of the frequency comb make it a particularly appealing source for molecular spectroscopy of human breath. The large bandwidth means that a single broad band comb can probe a large spectral range on its own, reducing the need for multiple light sources, and the individual comb lines allow for highly precise frequency measurement that can be comparable to using single-frequency lasers.[17] Combining this light source with a resonant high reflectivity cavity greatly increases the trace gas detection sensitivity of the comb-based spectrometer by repeatedly passing the light through the sample, increasing the strength of the absorption signal with each pass. Highly reflective mirrors can result in the beam effectively interacting with the sample several thousand times, providing the necessary absorption sensitivity required to detect extremely small (parts-per-billion, ppb to parts-per-trillion, ppt) sample concentrations. This technique is known as cavity-enhanced direct frequency comb spectroscopy (CE-DFCS).[17-21] In this application, our multidisciplinary team proposes to test this novel diagnostic tool, CE-DFCS, to address an important clinical conundrum. CE-DFCS is superior to mass spectrometry gas analysis techniques with higher sensitivity and no sample preparation needs. It exceeds other sensors with molecular identification capability by producing quantitative spectral data

of all the molecules contained in the breath. With further technology simplification this method also provides real-time diagnosis.

The specific aims of the study are to: 1. Differentiate breath patterns in children with two common respiratory diseases, asthma and viral CAP, that can both be caused by viral triggers; and 2. differentiate bacterial from viral etiology in children with CAP.

Intellectual Merit: The intellectual merit of using CE-DFCS to measure molecules in exhaled breath lies in its ability to provide highly precise, real-time, ultrasensitive measurements of molecular species at a level of detail previously unattainable with traditional methods. CE-DFCS generates a series of evenly spaced, narrow optical lines that can be precisely tuned to target specific molecular absorption features, allowing for the identification and quantification of gases and biomolecules in complex mixtures like exhaled breath from children. This technique offers significant advantages over commonly used clinical technologies, such as mass spectrometry, in terms of accuracy and sensitivity, enabling the detection of trace biomarkers associated with diseases such as asthma and potentially CAP. Furthermore, the combination of high-resolution spectroscopy with the non-invasive nature of breath analysis opens exciting possibilities for personalized medicine and early diagnostic tools, driving advancements in healthcare and molecular sensing. A patent has been filed by the University of Colorado (June 22, 2023, Title: Breath Analysis with Cavity-Enhanced Direct Frequency Comb Spectroscopy; CU Reference No.: CU5888B-PPA1) and a startup (Flari Tech Inc.) has been formed to commercialize this technology.

Interdisciplinary Team: ***CU Anschutz***: **Dr. Lilliam Ambroggio** (CUAnschutz PI) is an infectious disease epidemiologist and an Associate Professor in pediatrics with expertise in existing and emerging biomarkers for the diagnostics of CAP. She has extensive experience in conducting single-center and multi-center prospective studies.[39–41] **Dr. Brandie Wagner** is an Associate Professor of Biostatistics, and has expertise in analyzing microbiota communities from airway samples in lung diseases, identifying potential biomarkers to aid in determining the prognosis of patients with lung disease especially as it relates to infection. She will be designing and overseeing the statistical analysis as performed by **Mairead Dillon, MS**. **Dr. Eva Nozik** is a pediatric critical care physician and the Section Chief for the Section of Critical Care Medicine. Dr. Nozik's research program is to understand the contribution of redox regulated signaling to the regulation of pulmonary vascular structure and function, and to understand how disruption in these processes contributes to pediatric pulmonary vascular diseases. ***CU Boulder:*** **Dr. Jun Ye** (CU Boulder PI) is one of the original developers of OFC and the first to apply OFC in breath analysis.[29] He is a National Institute of Science and Technology scientist with his lab at JILA (a joint institute between NIST and UCB) located on the UCB campus. **Qizhong Liang** and Apoorva Bisht are Ph.D. candidates, and their Ph.D. research is to optimize the CE-DFCS for clinical capabilities. **Dr. David Nesbitt** is a NIST/JILA scientist and Adjoint Professor in the CU Chemistry and Physics departments, whose research program focuses on high resolution laser spectroscopy of radicals, chemical reaction dynamics, quantum nanostructures and photonic nanomaterials, and single molecule biophysics. ***Flari Tech Inc.*****: Dr. Xin Yao** is an entrepreneur with operating experience in diagnostics (non-invasive liquid biopsy) and CEO of Flari Tech Inc.

Methods Study Design The prospective cohort study leverages our existing infrastructure that was developed to ensure the feasibility of obtaining exhaled breath samples from children with CAP and asthma exacerbations, and to determine if any adverse events may occur with sample collection. We have enrolled 81 patients across all three groups to obtain the preliminary data for this proposal with no adverse events occurring. The study will enroll patients on the Hospital Medicine service at Children's Hospital Colorado. Potential participants are identified using EPIC by trained clinical research coordinators based on clinical diagnosis of asthma or viral or bacterial CAP. Consent is obtained from patients enrolled in the study, and exhaled breath samples are obtained. A viral CAP is defined as a child clinically diagnosed with CAP and a positive viral result on a Respiratory Pathogen Panel. A bacterial CAP is defined as a child clinically diagnosed with CAP and a positive atypical bacterial result on CAP or defervescence within 48 hours of receipt of antibiotic. An asthma exacerbation is defined as children who have an established diagnosis of asthma in the medical record and who present with wheeze and/or increased work of breathing that require albuterol and systemic corticosteroids. Participants exhale into a Tedlar bag to produce a breath sample. The breath sample is transported to the laboratory at UCB the same day for analysis. Through a patient survey and electronic

medical record review we will obtain demographic and clinical data. Participants will be compensated with a $25 gift card.

Molecular spectra data collection The breath sample is loaded into the OFC breathalyzer (into a cleaned vacuum chamber surrounded by a pair of optical mirrors). A frequency comb laser is coupled into the optical enhancement cavity to interact with the breath molecules. A Fourier Transform Infrared Spectrometer (FTIR) is used to measure the molecular absorption spectrum by determining the attenuation of light simultaneously at hundreds of thousands of optical frequencies. The spectrometer produces a time series recording of the optical power detected as a function of time or position, and this time series can be Fourier transformed to obtain a spectrum showing the frequency content of the transmitted light. In addition to 20 small molecules, we routinely examine for all breath samples (e.g., NO, CO_2, CO, N_2O, $OC^{18}O$, CH_4),[31] we will pay special attention to VOCs past research has investigated for asthma (CO, NO) and LRTI (e.g., acetone, nonanal, heptane) as potential candidates.[28]

Expected Outcomes Our aims are to differentiate breath patterns in children with bacterial LRTI, viral LRTI, and asthma exacerbation with a viral trigger. We will construct a prediction model capable of predicting each group based on molecular absorption patterns measured from exhaled breath. Sensitivity, specificity, positive predictive value, negative predictive value, and area under the receiver-operating characteristic curve, will be obtained for the prediction of each group. We will also identify a panel of important breath molecules for each group. Molecules that are identified would be those that have their concentrations changed significantly. This provides a way to elucidate the underlying pathophysiology in one group when compared to the other groups.

Statistical Analysis We will explore the data via volcano plots[22] and then apply Partial Least Squares-Discriminant Analysis (PLS-DA). PLS-DA is well-specified to spectroscopic data which contains a large amount of data channels (>1,000). PLS-DA and cross-validation methods will be used together to examine the prediction capability (e.g., receiver-operating-characteristic curve). Diagnostic accuracy and test characteristics will be reported. PLS-DA will produce a Variable Importance in Projection (VIP) score to identify a panel of exhaled breath molecules that strongly correlate with each group to assist in elucidating pathophysiological mechanisms. To account for multiple testing for the multiple VOCs we will use the false discovery rate procedure, Benjamini-Hochberg procedure.[23] We will also explore other machine learning techniques (e.g., random forest and support-vector-machines) to identify the best modeling strategy for the combined clinical and spectroscopic data to use for future studies.

Sample Size A minimum of 130 children per study group will allow for reliable estimates given 13 predictor variables (e.g., sex, age, days of illness) in the model. To ensure sufficient sample numbers, we plan to obtain consent, quality samples and data from ~130 children in each group (i.e., asthma, viral CAP, bacterial CAP). All analyses will be performed using SAS software version 9.4 or R (https://www.R-project.org/).

Potential Impact The optical frequency comb-based breath analyzer enabled with machine learning and under close collaboration with hospital medical teams will bring a wealth of molecular information related to medical conditions. Such information constitutes an expanding library of important biomarkers in human breath for the growing field of breath analysis. The unique knowledge we gain from this study will be shared with the wider medical community and can inform future designs and implementation of devices for differentiating the etiology of pediatric lower respiratory tract infections. In addition, Flari Tech Inc. is attempting to develop a portable medical device (benchtop breath analysis instrument) based on CE-DFCS. This device will be used for biomarker discovery as a research instrument, and a point-of-care diagnostic tool that can deliver breath-based tests at a price comparable to other high complexity molecular tests. With known biomarkers, Flari Tech Inc. will develop a device at the bedside and pursue regulatory approval. Therefore, this study is a pivotal study to support the ultimate commercialization of a frequency comb-based breath analysis device.

Long-Term Project Sustainability: Considering the innovation around technology and the multiple medical applications, we will seek funding from a diverse portfolio of funders including federal, foundation, and industry. The original development of CE-DFCS was supported by a grant from the Airforce, therefore as we continue to develop the technology for medical applications, we will seek future funding from the Department of Defense in addition to the National Institute of Health. Further, as we move further with the study, we anticipate Flari Tech Inc. to support future studies that are translational in nature.

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