Published in final edited form as:

Anal Chem. 2020 November 03; 92(21): 14594–14600. doi:10.1021/acs.analchem.0c02929.

Chemiluminescent Measurement of Hydrogen Peroxide in the Exhaled Breath Condensate of Healthy and Asthmatic Adults

Miguel E. Quimbar

BioLum Sciences LLC, Dallas, Texas 75206, United States

Steven Q. Davis, Sherif T. Al-Farra, Amanda Hayes, Valentina Jovic, Maximillian Masuda

John Peter Smith Hospital Network, Fort Worth, Texas 76104, United States

Alexander R. Lippert

BioLum Sciences LLC, Dallas, Texas 75206, United States; Department of Chemistry and Center for Drug Discovery, Design, and Delivery (CD4), Southern Methodist University, Dallas, Texas 75275-0314, United States

Abstract

Reactive oxygen species are centrally involved in the pathophysiology of airway diseases such as asthma and chronic obstructive pulmonary disease. This study reports the development of a chemiluminescence assay and a device for measuring hydrogen peroxide in the exhaled breath condensate of asthma patients and healthy participants. A stand-alone photon detection device was constructed for use with an optimized chemiluminescence assay. Calibrations using a catalase control to scavenge residual hydrogen peroxide in calibrant solutions provided analytically sensitive and specific measurements. We evaluated exhaled breath condensate hydrogen peroxide in 60 patients (ages 20–83; 30 healthy patients and 30 asthma patients) recruited from the John Peter Smith Hospital Network. The exhaled breath condensate hydrogen peroxide concentrations trended toward higher values in asthma patients compared to healthy participants (mean 142.5 vs 115.5 nM; $p = 0.32$). Asthma patients who had not used an albuterol rescue inhaler in the past week were compared to those who had and showed a trend toward higher hydrogen peroxide levels (mean 172.8 vs 115.9 nM; $p = 0.25$), and these patients also trended toward higher hydrogen peroxide than healthy participants (mean 172.8 vs 115.5 nM; $p = 0.14$). This pilot study demonstrates the ability of the newly developed assay and device to measure exhaled breath condensate hydrogen peroxide in asthma patients and healthy participants. The trends observed in this study are in agreement with previous literature and warrant further investigation of using this system to measure exhaled breath condensate hydrogen peroxide for monitoring oxidative stress in asthma.

Graphical Abstract

Corresponding Author: Alexander R. Lippert – BioLum Sciences LLC, Dallas, Texas 75206, United States; Department of Chemistry and Center for Drug Discovery, Design, and Delivery (CD4), Southern Methodist University, Dallas, Texas 75275-0314, United States; alippert@smu.edu.
Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Complete contact information is available at: <https://pubs.acs.org/10.1021/acs.analchem.0c02929>

INTRODUCTION

Oxidative stress is an important driver in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD)^{1,2} and is associated with increased exacerbations.³ During inflammation, white blood cells such as neutrophils, eosinophils, and macrophages undergo a rapid production of reactive oxygen species and reactive nitrogen species, including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite ($ONOO^-$), which results in increased levels of oxidative stress.^{4,5} Asthma phenotyping can be broken down into eosinophilic/type 2 asthma or non-eosinophilic/non-type 2 asthma that has a large neutrophilic component.^{6,7} Fractional exhaled nitric oxide (FeNO) is recommended for use in patients with eosinophilic asthma and is an invaluable tool in monitoring and managing the treatment of patients with asthma.⁷ Although exhaled nitric oxide can be used to differentiate type 2 from non-type 2 asthma, it is not as useful for monitoring neutrophilic-dominant asthma or COPD.^{8–10} There is currently an urgent need to develop therapies for neutrophilic asthma, but the lack of biomarkers and clinical methods to measure neutrophilic inflammation poses a critical obstacle.¹¹

Expanding the range of clinically detectable inflammatory biomarkers for monitoring neutrophilic inflammation will offer new opportunities to serve non-type 2 asthma patients with this severe and difficult-to-treat chronic disease. Hydrogen peroxide is relatively stable and can be detected in exhaled breath as a noninvasive biomarker of oxidative stress and can potentially track both eosinophilic and neutrophilic sources of production.^{12,13} When combined with FeNO, it should be possible to garner a precise and personalized view of patients' inflammation. Exhaled breath condensate (EBC) hydrogen peroxide is elevated in both adult^{14–22} and childhood^{23–27} asthma as well as in COPD.^{28–36} There is also a clinical potential for using exhaled hydrogen peroxide to monitor drug efficacy in asthma.^{17,22,37,38} In the exacerbations of COPD, exhaled hydrogen peroxide is elevated^{39,40} and can be used to track recovery from exacerbation.^{41–43} These studies highlight the potential for using hydrogen peroxide as a clinical biomarker, but improved measurement methods for use in a clinical setting are needed.

The measurement of hydrogen peroxide in biological systems^{44–49} and as a component in explosives⁵⁰ has been extensively studied. Enzymatic assays have been designed using horseradish peroxidase to catalyze reactions with hydrogen peroxide and a substrate to provide an observable optical or electrochemical response.⁵¹ For EBC samples, an absorbance-based method uses a colorimetric spectrophotometric assay consisting of 3',3',5',5'-tetramethylbenzidine in citrate buffer at pH 3.8 with horseradish peroxidase.^{17,19–21,27} A fluorometric assay with horseradish peroxidase and homovanillic acid²² or *p*-

hydroxyphenylacetic acid^{23,24} is also commonly used. Other methods include the d-ROM test, which uses the Fenton reaction to oxidize *N,N*-diethylparaphenyldiamine,³⁶ and the Amplex Red assay, which uses horseradish peroxidase to catalyze the generation of a fluorophore upon reaction with hydrogen peroxide.^{18,26,52} These optical methods can suffer from background absorbance and autofluorescence from interfering protein analytes that can be found in the EBC. Amperometric methods have also been used,²² but biofouling and calibration are significant challenges. All of these techniques require skilled technicians and expensive instrumentation, which have ultimately limited the feasibility of clinical use.

In order to address these limitations, we previously reported a peroxyoxalate chemiluminescent platform for measuring hydrogen peroxide in human EBC using a smartphone camera.⁵³ The chemiluminescent detection strategy uses an activated oxalate such as bis(2-carbopentyloxy-3,5,6-trichlorophenyl)oxalate (CPPO), an imidazole catalyst, and a dye that can be chemically excited by dioxetanedione formed from hydrogen peroxide (Scheme 1). This chemistry was shown to be analytically selective and was validated in human EBC samples by showing good agreement with the Amplex Red assay. The advantages of this system include good analytical sensitivity and high analytical specificity granted by a selective chemiluminescent reaction that does not require an external light source, dramatically reducing the background from autofluorescence and light scattering. Importantly, it does not require expensive spectrophotometers and is thus amenable to use at the point of care. In this study, we have expanded this method by the development of a stand-alone photon detection device for the measurement of hydrogen peroxide in EBC. In combination with market-approved EBC collection devices, we have used this method to measure hydrogen peroxide in a cohort of asthma patients and healthy volunteers.

EXPERIMENTAL SECTION

Reagents.

Rubrene, bis(2-carbopentyloxy-3,5,6-trichlorophenyl)oxalate, 95% (CPPO), and potassium permanganate were purchased from Alfa Aesar (Ward Hill, MA, USA). Imidazole was purchased from Acros Organics (Waltham, MA, USA). Catalase (bovine liver) was purchased from Millipore Sigma (Billerica, MA, USA). H₂O₂ (35%) was purchased from BeanTown Chemical (Hudson, NH, USA). All reagents were used as purchased without further purification. Ultrapure water (18.2 Ω, Milli-Q purification system, MilliporeSigma, Burlington, MA, USA) was used to make all H₂O₂ stock solutions. Sulfuric acid, acetone, and ethyl acetate were purchased from VWR (Radnor, PA, USA). The concentration of aqueous H₂O₂ was confirmed by redox titration in acid solution with a standard solution of potassium permanganate. A standard solution was made by dissolving 665 mg of potassium permanganate in 20 mL of ultrapure water and adding dropwise to an acidic solution of H₂O₂ until the endpoint was reached. The calculated concentration of the H₂O₂ bottle was 33.6% after storing at 4 °C in an opaque container for 11 weeks. The calculated rate of decay is 0.127% per week under the conditions listed above.

Recruitment and Protocol.

Fourteen male subjects and 46 female subjects (ages 20–83; mean age 50.1) were recruited from John Peter Smith Hospital (Fort Worth, TX, USA). Thirty asthmatic patients were recruited from an outpatient pulmonary clinic and 30 healthy controls were recruited from an outpatient sleep lab clinic. Exclusion criteria included patients that had consumed food or drink less than 1 h before the visit; smokers; former smokers who have smoked more than 5 pack years and quit less than 5 years prior to the study; patients who suffered upper respiratory, viral, or bacterial infection within 6 weeks of the visit; and those diagnosed with COPD, bronchitis, or other lung-related illnesses. Inclusion criteria included adults over the age of 18. After informed consent was obtained by the research staff, prospective study participants were verbally asked questions to confirm eligibility and record additional information. The questionnaire collected information on asthma diagnosis, albuterol use in the past month and week, scheduled physician visits for treatment, and long-term controller medication use. The study participants were tested using spirometry to assess pulmonary obstruction, and the following measurements were recorded: forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC), mean forced expiratory flow between 25 and 75% of the total expired volume (FEF 25–75%), forced expiratory volume in 3 s (FEV3), forced expiratory volume in 6 s (FEV6), forced expiratory flow (FEFmax), total expiration time (TET), and maximum voluntary ventilation (MVV). EBC was collected over 10 min of tidal breathing using the RTube EBC Collector and manufacturer's instructions. Nose clips were used during both spirometry and EBC collection by all participants. This protocol was approved by the North Texas Regional Institutional Review Board (IRB# 2018-131).

Collection of EBC.

EBC was collected using the market-approved RTube (Respiratory Research, Austin, TX, USA). The cooling sleeve was placed within a plastic bag to prevent excess moisture from freezing inside the tube and stored at -20°C for a minimum of 30 min before use. Nose clips were used to prevent nasal contamination and were well tolerated by all patients. Patients were instructed to exhale at tidal volume for 10 min with an allowance to pause momentarily to cough or sneeze. After collection, the samples were immediately extracted, transferred to labeled cryogenic tubes, and stored at -20°C until the end of the day, and then they were stored at -80°C until testing. All samples were tested within a week of collection.

Sensor Construction and Device Operation.

The device, herein called the BioSense 2.0 Laboratory Module, was custom-made to measure chemiluminescent signals in the visible spectrum (Figure 1). A surface-mounted silicon photodiode was used for data measurement and a Bluetooth 4.0 module is used for data transmission to a custom mobile phone application. Measurements were collected every 0.5 s over a period of 30 s. For this study, an adapter was designed (Solidworks 2018) and 3D-printed (CEL Robox 3D Printer, Portishead, Bristol, UK) to fit disposable semi-micro UV polypropylene cuvettes (BrandTech Scientific, Essex, CT, USA). To reduce the background light interference, five interior walls were covered in a double layer of aluminum sheets, and the sixth interior wall was covered with double layers of blue, green, red, orange, yellow, magenta, and purple gel color filters to block visible light. The BioSense

2.0 Laboratory Module lid was designed to activate a switch and start the reaction as it is being shut. The switch is engaged as the lid is almost closed, which can result in the first measurement from 0 to 0.5 s being exposed to ambient light and causing an overflow measurement above the limits of the sensor that is about 40 times brighter than the highest H_2O_2 concentration tested. Data are exported from the mobile application to a computer for analysis. The total analysis time for each measurement was 30 s with a total of 60 measurements. Because of the physical limitations of using manual operation to close the lid, the first measurement is disregarded in all tests, and only measurements 2–60 are used in the analysis.

Hydrogen Peroxide Chemiluminescent Assay.

All solutions were prepared daily and used within 3 h. Stock solutions of 7 mM rubrene, 60 mM imidazole, and 7 mM CPPO were prepared in 9:1 ethyl acetate/acetone and mixed via a vortexer until dissolved. An assay solution was made by preparing a solution of 3.4 mM rubrene, 3.2 mM imidazole, and 1.9 mM CPPO (300 μL volume) and incubating in a disposable semi-micro UV polypropylene cuvette (BrandTech Scientific, Essex, CT, USA) for 6 min. Each sample was thawed from $-80\text{ }^\circ\text{C}$ by allowing it to sit at room temperature for approximately 30 min before testing. The samples were measured by mixing 75 μL EBC (375 μL final volume) to the prepared assay solution and immediately beginning measurement with the newly developed device, herein called the BioSense 2.0 Laboratory Module. Interference was evaluated by incubating 1 μM H_2O_2 with 1 $\mu\text{g mL}^{-1}$ bovine serum albumin (BSA), 1 μM NaNO_2 , or at pH 6, 7, or 8 before and during measurement. Selectivity was evaluated by measuring the chemiluminescence response from 1 μM NaNO_2 , SIN-1, DEA NONOate, NaOCl , or $t\text{BuOOH}$.

Calibration.

A 5-point calibration was performed on the day of measurement, with H_2O_2 stock solutions of 0 nM, 100 nM, 250 nM, 500 nM, and 1 μM prepared with ultrapure water. The assay solution was prepared by gently mixing rubrene, imidazole, and CPPO by a pipette in a cuvette and incubating for 6 min at room temperature. A 75 μL of a given concentration of H_2O_2 stock solution was mixed with the incubated assay solution for each concentration and tested in triplicate. Light emission was measured over 30 s and averaged for each trial. The average values from the replicates at each concentration were plotted on a chart to calculate R^2 and slope values. A “catalase control” was achieved by measuring the light output from a sample of catalase-treated ultrapure water. The catalase control was performed with nine replicates, used as a standard to estimate H_2O_2 contamination, and subsequent calibration curves were corrected. The resulting plot was used to calculate the concentration of H_2O_2 samples by subtracting the y -intercept from the luminescent measurement and dividing by the slope of the plotted calibration curve. Statistical significance was defined as $p < 0.05$, as determined using the two-tailed Student's t test.

RESULTS AND DISCUSSION

Although our previous work provided an important proof of principle,⁵³ differences in the smartphone camera hardware and software for image acquisition prompted us to develop a

low-cost stand-alone photon detection device optimized for the point-of-care measurement of hydrogen peroxide in EBC (Figure 1). The reader is designed with a lid that can be opened, and a measured volume of sample can be inserted using a single-channel pipettor into an Eppendorf tube or cuvette by using appropriate 3D-printed adapters (Figure 1A). Upon closing the lid, photon detection from a photodiode is automatically initiated using an integrating amplifier circuit to provide a time course of the chemiluminescence emission over 30 s (Figure 1B). The data are transmitted via a Bluetooth connection to a custom application on a smartphone or a desktop computer. A photograph of the reader is shown in Figure 1C. This device can be used with the peroxyoxalate chemiluminescence assay shown in Scheme 1. During the measurement, the chemiluminescent precursor CPPO is first mixed with rubrene and the imidazole catalyst and incubated for 6 min. This incubation period is necessary to allow a small background luminescence to decay before making peroxide measurements.^{54,55} A sample can then be added and immediately inserted into the device for rapid measurement upon closing the lid. Hydrogen peroxide in the sample will react with CPPO to form dioxetanedione, which then reacts with rubrene in a chemically initiated electron exchange luminescence mechanism to produce rubrene in its excited state and initiate photon emission.⁵⁶ The response, interference, and sensitivity of the assay and the device were evaluated using 1 μM hydrogen peroxide. No large interferences were observed from 1 $\mu\text{g mL}^{-1}$ BSA, which is the concentration of protein found in EBC samples (Figure 2).⁵⁷ Preparing hydrogen peroxide solutions at pH 6, 7, or 8 also showed minimal interference. Selectivity was evaluated versus other reactive oxygen and nitrogen species, all of which showed no significant response, a result that is in agreement with our previous studies.⁵³

Calibrations and sample measurements were completed in triplicate, and the time course of the chemiluminescence emission from hydrogen peroxide shows a decay in emission over 30 s (Figure 3A). Calibrations were performed with increasing concentrations of hydrogen peroxide from 0 (vehicle control) up to 1000 nM hydrogen peroxide. It was noted that the vehicle control still displayed luminescence emission above baseline and higher than the instrument blank. We hypothesized that this could be due to residual hydrogen peroxide in the water used to prepare stock solutions for hydrogen peroxide calibration.⁵⁸ This was confirmed by performing a control experiment with the addition of 2 U mL^{-1} catalase, an enzyme that catalytically decomposes hydrogen peroxide (Figure 3B). Indeed, the addition of catalase reduces the signal down to the level of the instrument blank, where no sample is added. Using the catalase control as a true zero, calibration curves were adjusted to estimate the background concentration of hydrogen peroxide, which was found to be between 124 and 253 nM hydrogen peroxide, as independently measured on nine different days. The stock solution concentrations were then calculated by adding the background hydrogen peroxide concentration to the measured values (Figure 3C). The performance of the BioSense 2.0 Laboratory Module and assay was assessed for reproducibility and linearity. The limit of detection (LoD) was calculated as $3 \times \sigma_{\text{blank}}/\text{slope}$ using the catalase treated as zero for the blank signal. The linear range was between 0 and 1000 nM hydrogen peroxide. The average LoD across all testing days was 45 nM with a standard deviation of 12 nM, and the lowest LoD determined was 31 nM. The average R^2 value for all calibrations performed was 0.9900 with a standard deviation of 0.0133. The stability and reproducibility were

determined by measuring the same solution several minutes apart and determining the coefficient of variation (% CV = standard deviation/mean $\times$ 100%). The average (% CV) in these calibration measurements was found to be 8.4%.

This optimized method was used to investigate the ability of the point-of-care assay and device to measure hydrogen peroxide in the EBC of asthma patients and healthy participants (Figure 4). Table 1 shows the demographics of the participants in this study, who were recruited from the John Peter Smith Hospital system in Fort Worth, TX. A total of 60 patients were recruited, 30 of whom had a diagnosis of asthma and 30 of whom were recruited as healthy participants. Of the 30 asthma patients, 16 reported the recent use of an albuterol inhaler and 14 reported that they had not used the albuterol inhaler in the past 7 days. Upon recruitment, a sample of EBC was collected using an RTube as per the manufacturer's instructions. Generally, this involves tidal breathing over 10 min through a plastic tube cooled with a metal sleeve that is stored in a freezer between collections. This causes the breath to condense on the sides of the tube. The EBC can then be collected using a piston that pools the condensate upon scraping the sides of the tube. In most cases, approximately 1 mL of EBC is collected using this method. The samples were deidentified, and hydrogen peroxide measurements were made blind to the identity and asthma diagnosis of the participants. During the same visit for EBC collection, the participants were also evaluated using spirometry.

Representative raw data for EBC hydrogen peroxide are shown in Figure 4. In asthma patients that show elevated hydrogen peroxide (EBC-007), an increase in chemiluminescence emission intensity is observed during the first 15 s of measurement, with an exponential decay to baseline (Figure 4A). In contrast, in healthy participants with low levels of hydrogen peroxide (EBC-036), the chemiluminescence emission intensity is flat and closely resembles that of the catalase control experiment (Figure 4B). Averaging the emission intensity and comparing with a calibration curve from the same day of measurement provide a quantitative measurement of EBC hydrogen peroxide. A summary of the spirometry and EBC hydrogen peroxide measurements is given in Table 2. Spirometric assessments confirmed asthma diagnoses and showed significant differences between asthma patients and healthy participants. On average, the concentration of hydrogen peroxide in EBC trended toward higher values in asthma patients (142.5 nM) versus hydrogen peroxide values in healthy participants (115.5 nM). The highest average values of EBC hydrogen peroxide were observed in asthma patients that reported not using an albuterol inhaler in the past 7 days (172.8 nM), which trended higher than that of asthma patients that did report using the albuterol inhaler in the past 7 days (115.9 nM). Interestingly, there was no large difference in the average values of healthy participants (115.5 nM) and asthma patients with recent inhaler use (115.9 nM). Of these measures, the most significant difference was seen between asthma patients that reported no recent inhaler use and healthy participants (172.8 vs 115.5 nM, $p = 0.14$), but this did not reach the threshold of $p < 0.05$. It is important to note that the levels of EBC hydrogen peroxide are actually lower than what was found in ultrapure water, highlighting the presence of mechanisms for redox balance in the lungs.

CONCLUSIONS

We have reported the development of a point-of-care assay and a device for measuring hydrogen peroxide in EBC. The chemiluminescent measurement uses an optimized peroxyoxalate assay and a device that was constructed for low-cost measurement and ease of use. Importantly, we found that a control experiment using catalase to decompose any hydrogen peroxide present in the water used to prepare hydrogen peroxide stock solutions was critical for accurate calibration and measurement. The feasibility of using the assay and device in a clinical setting was established by measuring hydrogen peroxide concentrations in a cohort of asthma patients and healthy volunteers recruited from the John Peter Smith Hospital in Fort Worth, TX. Asthma diagnoses were supported by spirometry data, and the concentrations of hydrogen peroxide in the EBC were determined and found to be in agreement with the levels found in previous studies. A previous metastudy involving eight studies and 728 participants found that the hydrogen peroxide levels in both healthy participants and nonsevere asthma patients were in the nanomolar range.¹⁶ Our results are in line with these measurements. The weaknesses of the study include a relatively small sample size and a lack of stratification of asthma patients based on asthma control and severity. Although the values of exhaled hydrogen peroxide trended toward an increase in asthma patients, these did not reach a statistical significance of $p < 0.05$, likely due to this small sample size and lack of patient stratification. Nevertheless, the demonstrations of the use of this system in a clinical context pave the way forward to larger well-controlled studies to assess the usefulness of EBC hydrogen peroxide for monitoring and managing respiratory diseases.

ACKNOWLEDGMENTS

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R15GM114792-02. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional resources were provided by BioLum Sciences, LLC and the John Peter Smith Hospital.

The authors declare the following competing financial interest(s): M.E.Q. and A.R.L. disclose a financial stake in BioLum Sciences, LLC, which may benefit from the publication of this study. A patent application on the technology described in this study has been filed.

REFERENCES

- (1). Holguin F Ann. Am. Thorac. Soc 2013, 10, S150–S157. [PubMed: 24313766]
- (2). Mishra V; Banga J; Silveyra P Pharmacol. Therapeut 2018, 181, 169–182.
- (3). Drost EM; Skwarski KM; Sauleda J; Soler N; Roca J; Agusti A; MacNee W Thorax 2005, 60, 293–300. [PubMed: 15790984]
- (4). MacNee W Eur. J. Pharmacol 2001, 429, 195–207. [PubMed: 11698041]
- (5). Antczak A; Nowak D; Bialasiewicz P; Kasielski M Arch. Immunol. Ther. Exp 1999, 47, 119–126.
- (6). Chung KF; Wenzel SE; Brozek JL; Bush A; Castro M; Sterk PJ; Adcock IM; Bateman ED; Bel EH; Bleecker ER; Boulet L-P; Brightling C; Chanez P; Dahlen S-E; Djukanovic R; Frey U; Gaga M; Gibson P; Hamid Q; Jajour NN; Mauad T; Sorkness RL; Teague WG Eur. Respir. J 2014, 43, 343–373. [PubMed: 24337046]
- (7). Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2020. Available from: www.ginasthma.org. (accessed Oct 14, 2020).

- (8). Global Initiative for Asthma. Difficult-to-Treat & Severe Asthma in Adolescents and Adult Patients: Diagnosis and Management, 2019. Available from: www.ginasthma.com. (accessed Oct 14, 2020).
- (9). The Global Strategy for Diagnosis, Management and Prevention of COPD (updated 2020), the Pocket Guide (updated 2020) and the complete list of references examined by the Committee is available on the GOLD website. www.goldcopd.org. (accessed Oct 14, 2020).
- (10). Schneider A; Schwarzbach J; Faderl B; Welker L; Karsch-Völck M; Jörres RA *Respir. Med* 2013, 107, 209–216. [PubMed: 23107283]
- (11). Kalchiem-Dekel O; Yao X; Levine SJ *Chest* 2020, 157, 26–33. [PubMed: 31525357]
- (12). Fireman E; Shtark M; Priel IE; Shiner R; Mor R; Kivity S; Fireman Z *Inflammation* 2007, 30, 44–51. [PubMed: 17372840]
- (13). Antczak A; Nowak D; Bialasiewicz P; Kasielski M *Arch. Immunol. Ther. Exp* 1999, 47, 119–126.
- (14). Aldakheel FM; Thomas PS; Bourke JE; Matheson MC; Dharmage SC; Lowe AJ *Allergy* 2016, 71, 741–757. [PubMed: 26896172]
- (15). Thomas PS; Lowe AJ; Samarasinghe P; Lodge CJ; Huang Y; Abramson MJ; Dharmage SC; Jaffe A *Pediatr. Pulmonol* 2013, 48, 419–442. [PubMed: 23401497]
- (16). Teng Y; Sun P; Zhang J; Yu R; Bai J; Yao X; Huang M; Adcock IM; Barnes PJ *Chest* 2011, 140, 108–116. [PubMed: 21436249]
- (17). Loukides S; Bouros D; Papatheodorou G; Panagou P; Siafakas NM *Chest* 2002, 121, 338–346. [PubMed: 11834641]
- (18). Ueno T; Kataoka M; Hirano A; Iio K; Tanimoto Y; Kanehiro A; Okada C; Soda R; Takahashi K; Tanimoto M *Respiration* 2008, 13, 654–663.
- (19). Hamed A; Al Obaidi A *Ann. Thorac. Med* 2007, 2, 18–22. [PubMed: 19724670]
- (20). Emelyanov A; Fedoseev G; Abulimity A; Rudinski K; Fedoulov A; Karabanov A; Barnes PJ *Chest* 2001, 120, 1136–1139. [PubMed: 11591550]
- (21). Horváth I; Donnelly LE; Kiss A; Kharitonov SA; Lim S; Fan Chung K; Barnes PJ *Am. J. Respir. Crit. Care Med* 1998, 158, 1042–1046. [PubMed: 9769258]
- (22). Antczak A; Kurmanowska Z; Kasielski M; Nowak D *Respir. Med* 2000, 94, 416–421. [PubMed: 10868702]
- (23). Dohlman AW; Black HR; Royall JA *Am. Rev. Respir. Dis* 1993, 148, 955–960. [PubMed: 8214950]
- (24). Jöbsis Q; Raatgeep HC; Hermans PW; de Jongste JC *Eur. Respir. J* 1997, 10, 519–521. [PubMed: 9072978]
- (25). Trischler J; Merkel N; Könitzer S; Müller C-M; Unverzagt S; Lex C *Respir. Res* 2012, 13, 14. [PubMed: 22333039]
- (26). Caffarelli C; Calcinai E; Rinaldi L; Povesi Dascola C; Terracciano L; Corradi M *Respiration* 2012, 84, 291–298. [PubMed: 23018317]
- (27). Robroeks CMHHT; van de Kant KDG; Jöbsis Q; Hendriks HJE; van Gent R; Wouters EFM; Damoiseaux JGMC; Bast A; Wodzig WKWH; Dompeling E *Clin. Exp. Allergy* 2007, 37, 1303–1311. [PubMed: 17845410]
- (28). Nowak D; Kasielski M; Pietras T; Bialasiewicz P; Antczak A *Monaldi Arch. Chest Dis* 1998, 53, 268–273. [PubMed: 9785809]
- (29). Nowak D; Kasielski M; Antczak A; Pietras T; Bialasiewicz P *Respir. Med* 1999, 93, 389–396. [PubMed: 10464820]
- (30). De Benedetto F; Aceto A; Dragani B; Spacone A; Formisano S; Cocco R; Sanguinetti CM *Monaldi Arch. Chest Dis* 2000, 55, 185–188. [PubMed: 10948662]
- (31). Kostikas K; Papatheodorou G; Ganas K; Psathakis K; Panagou P; Loukides S *Am. J. Respir. Crit. Care Med* 2002, 165, 1364–1370. [PubMed: 12016097]
- (32). Kostikas K; Papatheodorou G; Psathakis K; Panagou P; Loukides S *Chest* 2003, 124, 1373–1380. [PubMed: 14555568]
- (33). Fireman E; Shtark M; Priel IE; Shiner R; Mor R; Kivity S; Fireman Z *Inflammation* 2007, 30, 44–51. [PubMed: 17372840]

- (34). Möller W; Heimbeck I; Weber N; Saba GK; Körner B; Neiswirth M; Kohlhäufel MJ *Aerosol Med. Pulm. Drug Deliv* 2010, 23, 129–135.
- (35). Inonu H; Doruk S; Sahin S; Erkorkmaz U; Celik D; Celikel S; Seyfikli Z *Respir. Care* 2012, 57, 413–419. [PubMed: 21968597]
- (36). Murata K; Fujimoto K; Kitaguchi Y; Horiuchi T; Kubo K; Honda T *COPD* 2014, 11, 81–87. [PubMed: 24111595]
- (37). Ganas K; Loukides S; Papatheodorou G; Panagou P; Kalogeropoulos N *Respir. Med* 2001, 95, 649–654. [PubMed: 11530952]
- (38). Ganas K; Loukides S; Papatheodorou G; Panagou P; Kalogeropoulos N *Respir. Med* 2001, 95, 649–654. [PubMed: 11530952]
- (39). Nagaraja C; Sagar BL; Manjunath P; Shashibhushan B; Asif M *Lung India* 2012, 29, 123–127. [PubMed: 22628925]
- (40). Dekhuijzen PN; Aben KK; Dekker I; Aarts LP; Wiolders PL; van Herwaarden CL; Bast A *Am. J. Respir. Crit. Care Med* 1996, 154, 813–816. [PubMed: 8810624]
- (41). Antczak A; Ciebiada M; Pietras T; Piotrowski WJ; Kurmanowska Z; Górski P *Arch. Med. Sci* 2012, 2, 277–285.
- (42). Gerritsen WBM; Asin J; Zanen P; Bosch J. M. M. v. d.; Haas FJLM *Respir. Med* 2005, 99, 84–90. [PubMed: 15672854]
- (43). Oudijk E-JD; Gerritsen WBM; Nijhuis EHJ; Kanters D; Maesen BLP; Lammers J-WJ; Koenderman L *Respir. Med* 2006, 100, 1791–1799. [PubMed: 16531033]
- (44). Zappacosta B; Persichilli S; Mormile F; Minucci A; Russo A; Giardina B; De Sole P *Clin. Chim. Acta* 2001, 310, 187–191. [PubMed: 11498084]
- (45). Rios N; Radi R; Kalyanaraman B; Zielonka JJ *Biol. Chem* 2020, 295, 6665–6676.
- (46). Lippert AR; Keshari KR; Kurhanewicz J; Chang CJ *J. Am. Chem. Soc* 2011, 133, 3776–3779. [PubMed: 21366297]
- (47). Lippert AR; Van De Bittner GC; Chang CJ *Acc. Chem. Res* 2011, 44, 793–804. [PubMed: 21834525]
- (48). Ye S; Yang D; Hananya N; Green O; Chen H; Zhao AQ; Shen J; Shabat D *Angew. Chem. Int. Ed* 2020, 59, 14326–14330.
- (49). Hardy M; Zielonka J; Karoui H; Sikora A; Michalski R; Podsiadly R; Lopez M; Vasquez-Vivar J; Kalyanaraman B; Ouari O *Antioxid. Redox Signaling* 2018, 28, 1416–1432.
- (50). Burks RM; Hage DS *Anal. Bioanal. Chem* 2009, 395, 301–313. [PubMed: 19644679]
- (51). Regalado C; García-Almendárez BE; Duarte-Vázquez MA *Phytochem. Rev* 2004, 3, 243–256.
- (52). Zhou M; Diwu Z; Panchuk-Voloshina N; Haugland RP *Anal. Biochem* 1997, 253, 162–168. [PubMed: 9367498]
- (53). Quimbar ME; Krenek KM; Lippert AR *Methods* 2016, 109, 123–130. [PubMed: 27233749]
- (54). Williams DC III; Huff GF; Seitz WR *Anal. Chem* 1976, 48, 1003–1006. [PubMed: 1267167]
- (55). Van Zoonen P; Kamminga DA; Gooijer C; Velthorst NH; Frei RW *Anal. Chim. Acta* 1985, 167, 249–256.
- (56). Vacher M; Galván IF; Ding B-W; Schramm S; Berraud-Pache R; Naumov P; Ferré N; Liu Y-J; Navizet I; Roca-Sanjuán D; Baader WJ; Lindh R; Lindh R *Chem. Rev* 2018, 118, 6927–6974. [PubMed: 29493234]
- (57). Bloemen K; Lissens G; Desager K; Schoeters G *Respir. Med* 2007, 101, 1331–1337. [PubMed: 17125983]
- (58). Choi J; Chung J *Anal. Sci* 2020, DOI: 10.2116/analsci.20A003. . Published online: July 24, 2020. https://www.jstage.jst.go.jp/article/analsci/advpub/0/advpub_20A003/_article (accessed Sep 30, 2020)

Figure 1.

Design of a point-of-care device for the detection of hydrogen peroxide in EBC. (A) Schematic of the device in the open position where the sample can be inserted by a pipette. (B) Schematic of the device in the closed position. Closing the lid instantly initiates a time course of photon detection and transmits data to a smartphone application using Bluetooth communication. (C) Photograph of the point-of-care device for measuring hydrogen peroxide in EBC.

Figure 2.

Interference and selectivity of the assay containing 3.4 mM rubrene, 3.2 mM imidazole, and 1.9 mM CPPO. All analytes were tested at 1 μ M. Bars are the chemiluminescence emission intensities at 1 s (red bar) and 5, 10, 20, and 30 s (black-gray bars).

A Calibration Raw Data **B Vehicle Control and Instrument Blank** **C Calibration Curve** **Figure 3.**

Calibration for quantitative hydrogen peroxide determination using the device described in Figure 1. (A) Time course of the chemiluminescence emission upon the addition of 75 μL of 0 (vehicle control), 100, 250, and 500 nM of aqueous H_2O_2 solutions to 300 μL of a solution containing 1.9 mM CPPO, 3.2 mM imidazole, and 3.4 mM rubrene in 9:1 ethyl acetate/acetone. The three traces are from three independent replicates of a sample. (B) Time course of the chemiluminescence emission of the vehicle control with the addition of 2 U mL^{-1} catalase and comparison with the instrument blank. (C) Correction of the calibration curve by the determination of the hydrogen peroxide concentration in the vehicle control.

Figure 4. Representative raw measurements in (A) an asthma patient with elevated EBC hydrogen peroxide and (B) a healthy participant with low EBC hydrogen peroxide. The three traces are from three independent replicates of a sample.

Scheme 1.
Chemiluminescent Peroxyoxalate Assay for Hydrogen Peroxide Detection

Table 1.

Patient Population and Characteristics

characteristic	total (n = 60)	asthma (n = 30)	healthy (n = 30)	asthma with albuterol ^a (n = 14)	asthma without albuterol ^b (n = 14)
age (years)—mean (SD)	50.3 (12.0)	52.1 (13.0)	48.6 (10.9)	55.8 (12.8)	58.1 (22.8)
sex					
male—mean (%)	14 (23%)	5 (17%)	9 (30%)	2 (12%)	3 (21%)
female—mean (%)	46 (77%)	25 (83%)	21 (70%)	14 (88%)	11 (79%)
height (in.)—mean (SD)	64.9 (3.68)	64.5 (4.05)	65.3 (3.29)	64.6 (4.5)	64.3 (3.63)
weight (lbs)—mean (SD)	246.3 (89.69)	248.3 (102.9)	244.3 (75.96)	257.1 (84.6)	238.1 (123.1)
BMI	40.8 (13.3)	41.5 (14.6)	40.1 (12.0)	43.3 (13.5)	39.4 (16.1)
ethnicity—n (%)					
African American	26 (43%)	11 (37%)	15 (50%)	7 (43%)	4 (29%)
Asian	2 (3%)	1 (3%)	1 (3%)		1 (7%)
Caucasian	16 (27%)	11 (37%)	5 (17%)	6 (38%)	5 (36%)
Hispanic	14 (23%)	7 (23%)	7 (23%)	3 (19%)	4 (29%)
other	2 (3%)		2 (7%)		

^a Asthma patients who reported using an albuterol rescue inhaler at least once within the past 7 days.

^b Asthma patients who reported not using an albuterol rescue inhaler within the past 7 days.

Table 2.

Spirometry and EBC Hydrogen Peroxide Data

assessment	total (<i>n</i> = 60)	asthma (<i>n</i> = 30)	healthy (<i>n</i> = 30)	asthma with albuterol ^a (<i>n</i> = 16) ^c	asthma without albuterol ^b (<i>n</i> = 14)
			Spirometry		
FEV1 (L)—mean (SD)	2.04 (0.86)	1.71 (0.87)	2.35 (0.71)	1.85 (0.69)	1.66 (0.76)
FEV1 % predicted, mean, %	75.4% (26.3%)	66.0% (25.0%)	84.0% (57.5%)	72.3% (22.8%)	62.2% (26.3%)
FVC (L)—mean (SD)	2.64 (1.02)	2.28 (0.92)	2.98 (1.00)	2.44 (0.94)	2.21 (0.95)
FVC % predicted, mean, %	72.7% (21.6%)	65.2% (20.8%)	79.7% (20.2%)	69.3% (17.0%)	61.9% (23.9%)
FEV1/FVC ratio—mean (SD)	76.8 (10.9)	75.0 (10.6)	78.5 (10.6)	76.4 (11.7)	75.1 (10.8)
FEV1/FVC ratio % predicted, mean, %	102.3% (16.1%)	100.6% (16.0%)	103.9% (16.3%)	103.8% (15.7%)	98.8% (15.6%)
FEF25–75%—mean (SD)	2.12 (1.09)	1.67 (0.58)	2.53 (1.05)	1.93 (0.97)	1.57 (0.84)
FEF25–75% % predicted, mean, %	70.2% (34.1%)	57.8% (33.4%)	81.4% (31.2%)	68.1% (36.2%)	52.3% (26.9%)
FEV3 (L)—mean (SD)	2.46 (0.97)	2.11 (0.88)	2.78 (0.96)	2.30 (0.88)	2.02 (0.85)
FEV3% predicted, mean, %	69.8% (21.6%)	62.3% (21.1%)	76.5% (20.0%)	67.5% (18.3%)	58.6% (23.0%)
FEV3/FVC, L, mean, (SD)	92.8 (5.91)	92.3 (6.82)	93.3 (5.00)	93.9 (6.88)	91.6 (6.31)
FEV3/FVC % predicted, mean, %	95.6% (6.10%)	95.1% (6.96%)	96.0% (5.26%)	96.8% (6.88%)	94.4% (6.58%)
FEV6 (L)—mean (SD)	2.61 (1.01)	2.24 (0.92)	2.95 (0.98)	2.41 (0.95)	2.17 (0.93)
FEFmax (L)—mean (SD)	4.08 (1.92)	3.38 (1.41)	4.72 (2.12)	3.72 (1.32)	3.09 (1.47)
FEFmax % predicted, mean %	60.9% (27.3%)	52.1% (21.3%)	68.8% (30.1%)	57.2% (20.2%)	47.2% (22.0%)
TET (s)—mean (SD)	7.01 (0.96)	7.17 (1.10)	6.86 (0.80)	6.93 (1.19)	7.32 (1.07)
MVV (L)—mean (SD)	105.3 (19.1)	102.5 (20.5)	107.9 (17.6)	101.4 (21.5)	106.3 (20.3)
			H ₂ O ₂		
H ₂ O ₂ (nM)—mean (SD)	129.0 (103.3)	142.5 (134.2)	115.5 (57.7)	115.9 (37.3)	172.8 (191.5)

^a Asthma patients who reported using an albuterol rescue inhaler at least once within the past 7 days.

^b Asthma patients who reported not using an albuterol rescue inhaler within the past 7 days.

^c *n* = 14 for spirometry assessments.