Original Article on Air Pollution

Malondialdehyde in dried blood spots: a biomarker of systemic lipid peroxidation linked to cardiopulmonary symptoms and risk factors

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Background: There are few oxidative biomarkers that can be used in resource-limited settings (e.g., rural Africa) where blood collection facilities are lacking. This study aims to evaluate the potential of malondialdehyde (MDA) in dried blood spots (DBS) as a useful biomarker to monitor cardiopulmonary health.

Methods: We first conducted a cross-validation comparison of matched capillary DBS, plasma, and whole venous blood collected from nine healthy volunteers for the measurement of total MDA (free + conjugated) and C-reactive protein (CRP), a well-established biomarker of systemic inflammation. Then a field study was conducted in a rural Senegal with a population of 441 women routinely exposed to severe household air pollution, examining associations of MDA and CRP levels in 882 DBS with self-reported cardiopulmonary symptoms.

Results: In the cross-validation study, CRP levels were strongly correlated across DBS, plasma, and whole blood. MDA levels were correlated between DBS and whole blood and were 1–2 orders of magnitude lower in plasma, suggesting that DBS MDA may reflect total oxidation levels in intracellular and extracellular compartments. In the field study, we observed significantly higher MDA levels in women with secondhand smoke exposure. An interquartile range increase in MDA concentration was associated with 27.0% (95% CI: 3.1–56.5%) and 21.1% (95% CI: –3.5% to 52.0%) increases in the incidence of chest tightness and breath difficulty, respectively. In contrast, CRP levels were not associated with worse outcomes or risk factors.

Conclusions: These results support the use of DBS as a convenient alternative to venous blood when MDA is measured as a biomarker for cardiopulmonary health risk.

Keywords: Malondialdehyde; dry blood spot; C-reactive protein (CRP); oxidative stress; cardiopulmonary symptoms

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Introduction

Systemic inflammation and oxidation are key early events in the pathophysiology of cardiopulmonary diseases. The detection of these events relies on the availability of blood biomarkers. For example, increased circulating levels of pro-inflammatory [e.g., C-reactive protein (CRP)] and oxidative [e.g., malondialdehyde (MDA)] biomarkers have been documented in patients with asthma (1,2), chronic obstructive pulmonary disease (3,4), hypertension (5,6), and ischemic heart disease (7,8). CRP and MDA levels are also responsive to changes in environmental factors or stressors such as air pollution (9,10). Linked to both disease development and environmental risk factors, circulating levels of CRP and MDA have thus been widely used in population studies as early biomarkers for cardiopulmonary health risk. However, most prior studies have been conducted in high- and middle-income countries, while data from low-income countries and settings (e.g., sub-Saharan African countries) are relatively scare.

A major obstacle restricting the use of circulating CRP and MDA levels in large population studies in rural areas of low-income countries is the requirement for venous blood. Blood collection requires a trained phlebotomist to perform invasive venipuncture, as well as accessible facilities where blood samples can be processed in a timely fashion. Importantly, a phlebotomist or facilities may not be readily available in remote areas where such samples would be of particular value, and the complex and inconvenient procedure that is required may decrease willingness of members of the target population to participate. As an alternative to blood samples, dried blood spot (DBS) specimens can be self-collected via finger pricking, blotting and drying on filter papers for convenient storage and shipment. Over the past several decades, DBS have been increasingly common in population studies, especially in non-clinical settings or remote field sites (11), and a growing number of DBS biomarkers (e.g., CRP) have been developed and validated (12,13). However, no studies have measured MDA levels in DBS in the context of cardiopulmonary health risk assessment.

In this study, we evaluate the feasibility of using MDA in DBS as a biomarker of oxidative stress and cardiopulmonary health risks. Toward this goal, we first perform a cross-validation study in nine healthy volunteers to compare the levels of MDA in DBS, plasma, and whole blood samples. The levels of CRP, a previously validated DBS biomarker (12,13), were also measured for the purpose of comparison. Additionally, we performed a field-validation study in 441 rural women in central and northern Senegal who are regularly exposed to household air pollution, examining the associations of CRP and MDA levels in DBS with self-reported cardiopulmonary health symptoms. We present the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/jtd-21-604).

Methods

Study participants and sample collection

This study consisted of cross-validation and field-validation components, both of which conformed to the provisions of the Declaration of Helsinki (as revised in 2013) and were approved by the institutional review board of Duke University (Protocols 2018-0061 and 2020-0297). All participants gave their informed consent to participate prior to measurements. The aim of the cross-validation component was to compare the concentrations of CRP and MDA across different types of blood specimens. We recruited nine healthy volunteers (six men and three women, ages 18–55) at Duke University, and collected paired plasma, whole venous blood, and capillary DBS (cDBS) samples. Plasma samples were collected with tubes containing K_2-EDTA anticoagulant (BD Bioscience, San Jose, CA, USA) and subsequently separated from blood cells within 1 hour of collection. The cDBS samples were collected with a finger prick, and blood drops were collected with a Whatman 903 Protein Saver card. For each participant, we collected four spots with a single finger prick, noting their chronological order. The whole venous blood was also spotted to a Whatman 903 Protein Saver card in order to prepare venous DBS (vDBS). The cDBS and vDBS samples were dried at room temperature for ~18 hours, and stored in sealed plastic bags at –20 Celsius until they were analyzed.

The aim of the field-validation component was to determine the levels of CRP and MDA biomarkers in DBS collected from women primary cooks in rural Senegal to assess their relationship with cardiopulmonary health indicators. Participants (n=485) were recruited from 15 villages located in the regions of Saint-Louis (eight villages) in northern Senegal and Kaffrine (seven villages) in central Senegal. A common feature of these participants is that they were exposed to severe household air pollution from solid fuel use during cooking with average 24-hour

concentrations of fine particles ($PM_{2.5}$) in their kitchens of 530 μg/m^3. These regions were selected due to their low penetration of improved and clean cooking technologies. Within a region, villages were randomly selected from the full set of villages in the two regions, stratified by department to ensure coverage within each region. In each village, households were randomly selected to participate in the study using a field-based counting method. Selected households completed baseline surveys administered by trained enumerators, who also coordinated two on-site visits for each participant in 2018 and 2019 to collect cDBS samples and survey on cardiopulmonary symptoms (i.e., breath difficulty, chest tightness, and cough/cold in the past two weeks) and exposures to secondhand smoke. The cDBS samples were collected with a finger prick using a disposable safety lancing device, and blood drops were collected with a Whatman 903 Protein Saver card that was then stored in a freezer as soon as possible. All measurements were taken by certified Senegalese nurses with additional training on safety and hygiene regulations.

Laboratory analysis

We extracted analytes from the DBS samples based on a previously established protocol with modifications (14). Briefly, two pieces of cDBS or vDBS samples were punched with a 3 mm Miltex disposable biopsy punch, and were incubated with 150 μL phosphate buffered saline buffer containing 0.1% Triton X100 and 0.03% Tween-20 on a shaker for 30 minutes. The mixture was transferred to a Spin-X tube and centrifuged at 8,000 g for 10 minutes, and then the blood elute was collected. We performed an additional wash with 50 μL buffers and the final elute volume was 200 μL.

To determine the levels of total (unconjugated + conjugated) MDA (tMDA), 20 μL DBS extract or diluted plasma samples (1:100, v/v) was mixed with 65 μL 1N NaOH in a 1.5-mL screw cap tubes and incubated at 60 ℃ for 30 min. After the reaction, 65 μL 1N HCl, 750 μL phosphoric acid and 150 μL TBA solution were added to each vial. The mixture was incubated in the oven at 80 ℃ for 1 hour and then cooled to room temperature before being injected into the Waters 2695 HPLC system with a multi λ fluorescence detector (Waters 2475). The analyte was separated with a Hypersil Gold column (C8, 5 μm, 4.6×150 mm, Waters). The mobile phase consisted of 40% (v/v) methanol and 60% (v/v) 50 mM KH_2PO_4 solution with its pH adjusted to 6.8. The flow rate was 0.8 mL/min, and the injection volume was 20 μL. The excitation and emission wavelength lengths were 532 and 553 nm, respectively. The level of CRP in DBS extract or plasma samples was measured with a commercial ELISA kit (The Cayman Chemical). The level of hemoglobin in DBS extract was measured with spectrophotometry at a wavelength of 410 nm (15).

Statistical analysis

We examined the normality of each biomarker with Shapiro-Wilk test, and tabulated mean ± standard deviations for variables with normal distributions and geometric mean [interquartile range (IQR)] otherwise. In the cross-validation analysis, we used linear mixed effects models with random intercepts for participant levels to examine whether the chronological order of blood drops influenced biomarker levels in cDBS. The associations between biomarker levels in different sample types (i.e., plasma, cDBS, and vDBS) were tested with linear regression. In the field-validation analysis, we compared biomarker concentrations in different population subgroups with univariate linear mixed effects models with random intercepts of participants. The associations of biomarker concentrations with self-reported cardiopulmonary symptoms (dichotomous variables) were tested by logistic mixed effects models controlling for random participant intercepts, and fixed effects of secondhand smoke exposure, and history of asthma, cardiovascular diseases, and high blood pressure. Alpha was set at 0.05, and all tests were two-tailed. All data analysis was performed in statistical software R (version 3.3.2, www.r-project.org).

Results

Cross-validation study

Hematocrit levels have been previously identified as the most significant parameter affecting blood spot characteristics and assay reproducibility (11,16). Therefore, we first investigated the effects of hematocrit variations on biomarker measurement by comparing four different cDBS samples collected from the same finger prick (*Figure 1A*,*B*,*C*,*D*). We observed a decreasing trend in unadjusted concentrations of CRP and tMDA from the first to the fourth cDBS samples collected from a single finger prick (*Figure 1A*,*C*, $P<0.05$). A decreasing trend was similarly observed for hemoglobin concentrations in cDBS

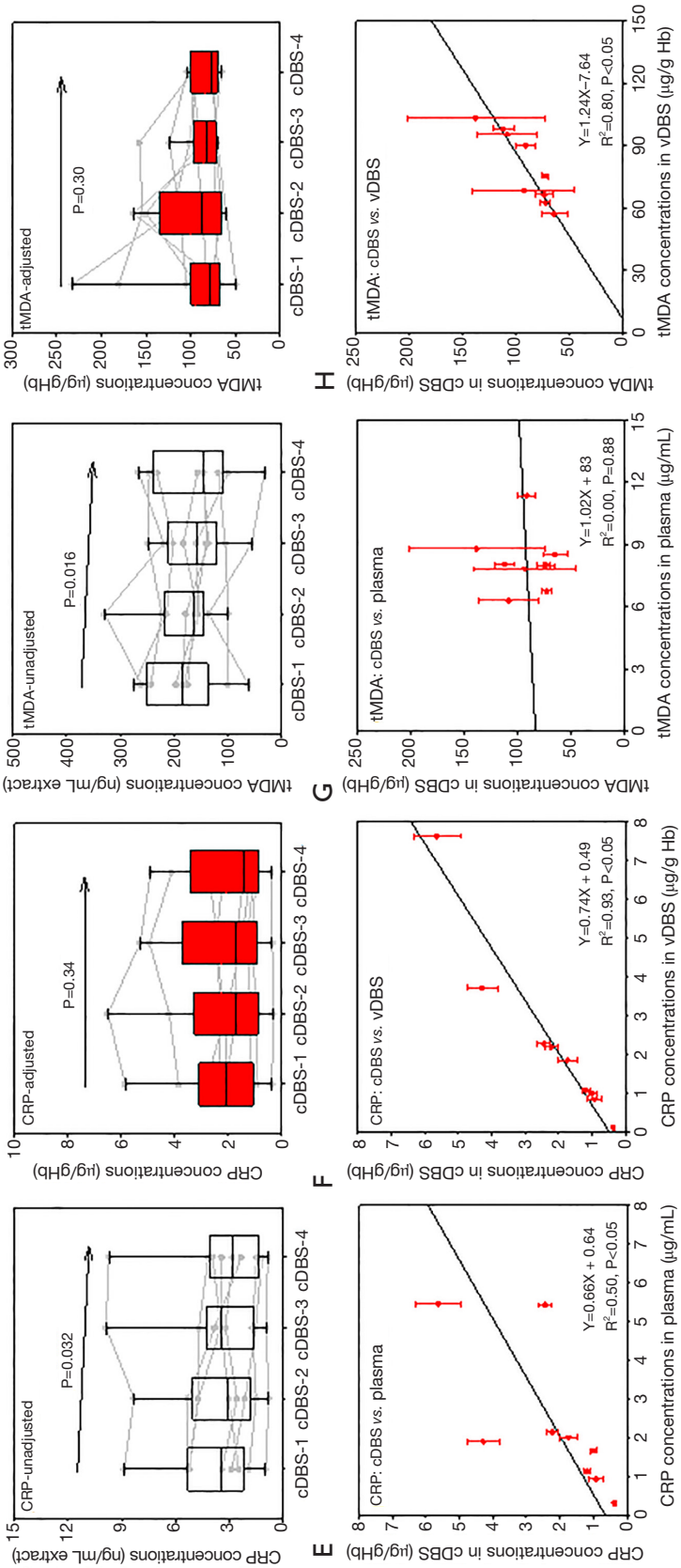

Figure 1 Unadjusted and hemoglobin-adjusted cDBS concentrations of CRP (A,B) and tMDA (C,D) in different blood drops from the same participants with a single finger prick, as well as the relationship of CRP (E,F) and tMDA (G,H) concentrations in plasma, vDBS, and cDBS samples. White and red plots indicate unadjusted and adjusted data, respectively. $P<0.05$ in panels A-D indicates significant decreasing trends of biomarker concentrations in a chronological order as tested using linear mixed effects models with random intercepts for study participants. Biomarker concentrations of cDBS samples in panels E-H are average levels of four spots collected from the same participants, and the error bars indicate standard deviations. CRP, C-reactive protein.

Table 1 Baseline characteristics of study participants (N=441)

Characteristic	Mean ± standard deviation (or N)	Range (or %)
Age	32.1±9.7	15–73
Ethnicity		
Poulard	103	23.4
Wolof	322	73.0
Others	16	3.6
Education		
Religious school	133	30.2
< Middle school	289	65.5
Middle or high school	16	3.6
> High school	3	0.7
History of asthma		
No	386	87.5
Yes	21	4.8
Not reported	34	7.7
History of cardiovascular diseases		
No	368	83.4
Yes	39	8.8
Not reported	34	7.7
History of high blood pressure		
No	197	44.7
Yes	207	46.9
Not reported	37	8.4
District		
Birkelane (in Kaffrine)	30	6.8
Kaffrine (in Kaffrine)	91	20.6
Koungheul (in Kaffrine)	81	18.4
Dagana (in Saint Louis)	119	27.0
Podor (in Saint Louis)	60	13.6
Saint Louis (in Saint Louis)	60	13.6

(Figure S1, P=0.06). Hemoglobin-normalized CRP and tMDA concentrations no longer exhibited a significant trend among the four batches of samples (*Figure 1B*,*D*). Hence, we normalized CRP and MDA concentrations by hemoglobin level in all subsequent analyses.

We found that CRP levels were significantly correlated across cDBS, vDBS, and plasma samples (*Figure 1E*,*F* and Figure S2, P<0.05). In contrast, the tMDA levels were only correlated between cDBS and vDBS samples, and the plasma tMDA levels were not correlated with those in cDBS or vDBS samples (*Figure 1G*,*H*, and Figure S2). Remarkably, despite comparable levels of CRP (range, 0.33–5.4 μg/mL) and tMDA (range, 6.3–11.4 μg/mL) in plasma, the level of tMDA in cDBS (range, 72.2–137 μg/g hemoglobin) was higher by more than one order of magnitude than that of CRP (range, 0.92–5.63 μg/g hemoglobin). These results suggest marked contributions of tMDA in the non-plasma fraction, likely in conjugated forms bonded to intracellular proteins such as hemoglobin (17), to the tMDA concentrations measured in DBS samples.

Field study

Out of the 970 designated cDBS from 485 participants, 925 (95.3%) samples were successfully collected with 43 participants missing one collection and one participant missing both collections. Such high levels of compliance speak to the acceptability and feasibility of DBS collection in remote and low-income contexts, and all samples were successfully shipped, without any special cryogenic treatment, to Duke University in the US for biomarker measurements. The current analysis includes 882 cDBS samples collected from 441 participants who completed both collections. Of the 441 participants, 4.8%, 8.8%, and 46.9% have a self-reported medical history of asthma, cardiovascular diseases, and high blood pressure, respectively. The average (standard deviations) age of these 441 participants was 32.1 (9.7) years, and most of them identified as Poulard or Wolof (*Table 1*). Only 4.3% of the participants had attended middle school or received higher education.

While all the cDBS samples were punched with a 3-mm disposable biopsy punch for biomarker analysis, 120 out of 882 (13.6%) samples had blood spots with diameters less than 3-mm. As expected, unadjusted concentrations of CRP and tMDA in these 120 samples were significantly lower than the others (Figure S3). Nevertheless, there is no significant difference in hemoglobin-normalized concentrations of tMDA between the two groups of cDBS samples, and the hemoglobin-normalized concentrations of CRP were otherwise higher in 120 samples with inadequate blood (Figure S3). These results further support

Table 2 Concentrations of CRP and tMDA in cDBS collected from women in rural Senegal

Subgroups	Number of samples	CRP (μg/g hemoglobin)	tMDA (μg/g hemoglobin)
All	882	2.39 (1.02–6.16)[a]	80 (59–131)
Visit			
Visit 2018 (reference)	441	2.14 (0.94–5.25)	106 (91–155)
Visit 2019	441	2.67 (1.09–6.76)[b]	61 (45–88)[b]
Exposures to secondhand smoke			
No (reference)	854	2.39 (1.01–6.17)	80 (59–130)
Yes	28	2.29 (1.43–3.97)	101 (81–142)[b]

[a], geometric mean (interquartile range); [b], significantly different from reference group (P<0.05) tested by mixed effect models with random intercept of participants. CRP, C-reactive protein; tMDA, total (unconjugated + conjugated) malondialdehyde; cDBS, capillary dried blood spots.

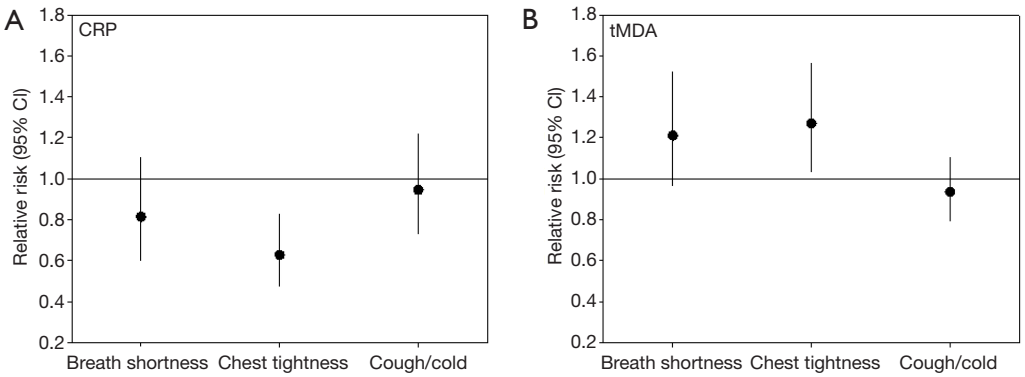

Figure 2 Associations of CRP (A) and tMDA (B) levels in cDBS with the incidence of cardiopulmonary symptoms in past two weeks. Relative risks of cardiopulmonary symptoms associated with one IQR increases in biomarker concentrations were tested using logistic mixed effects models with random intercepts for study participants, controlling for the fixed effects of secondhand smoke exposures and histories of asthma, cardiovascular diseases, and high blood pressure. CRP, C-reactive protein; tMDA, total (unconjugated + conjugated) malondialdehyde; cDBS, capillary dried blood spots.

the usefulness of hemoglobin normalization to reduce the influence of unmeasured variation in blood volume on DBS biomarker concentrations.

The geometric mean [IQR] tMDA and CRP concentration in cDBS of the participants was 80 [59–131] and 2.39 [1.02–6.16] μg/g hemoglobin, respectively. The tMDA level was significantly higher in participants with self-reported exposure to secondhand smoke (P<0.05, *Table 2*). In contrast, no significant difference in CRP levels was observed between participants with and without secondhand smoke (*Table 2*). We found that an interquartile range increase in tMDA level was significantly associated with 27.0% (95% CI: 3.1–56.5%) increases in the risk of chest tightness (*Figure 2*). In addition, a borderline increase in the risk of breath difficulty (relative risk: 1.21, 95% CI: 0.97–1.52, P=0.098) was also observed in association with increased tMDA levels (*Figure 2*). We did not observe any positive association between CRP levels and cardiopulmonary symptoms. To our surprise, we found a negative association between CRP levels and the risk of chest tightness (relative risk: 0.63, 95% CI: 0.48–0.83, P<0.001).

Discussion

The heavy burden of cardiopulmonary diseases in resource-limited populations elevates the need for biomarkers using specimens that can be conveniently collected and handled in the field. In this study, we provide cross-validation evidence supporting MDA in DBS as a biomarker of total

systemic lipid peroxidation. We further show that DBS MDA concentrations, normalized by hemoglobin levels, are positively associated with cardiopulmonary symptoms in 441 rural Senegal women who are exposed to high levels of household air pollution. Our exploratory findings indicate that DBS MDA may serve as a convenient and sensitive biomarker for cardiopulmonary health risk.

Blood hematocrit has been shown to markedly affect the concentrations of DBS biomarkers, especially when a fixed area was punched for analysis (11,16). In our study, results from both cross-validation and field studies suggested that the normalization of hemoglobin levels would reduce the hematocrit bias, which is supported by existing knowledge on how hematocrit affects DBS characteristics. On the one hand, increased hematocrit level leads to higher blood viscosity and therefore restricts blood diffusion on filter paper. This results in a larger volume of blood for the measurement when a sub-sample disk punch is analyzed (16). On the other hand, DBS consists of tissue fluid, plasma, and erythrocytes. An increased proportion of tissue fluid will decrease the actual volume of blood punched for analysis, and will thus be accompanied by decreased hematocrit levels. Previous studies have found that the addition of tissue fluids may be caused by milking or squeezing the puncture (18), which is not recommended during sample collection, but may occur especially when multiple blood spots are collected from participants. Because hemoglobin levels serve as an objective measure of blood volume, the data normalization by hemoglobin reduces the influence of hematocrit level on biomarker concentrations.

MDA exists in both unconjugated and conjugated forms. In the conjugated form, MDA is covalently bonded to the -SH and -NH_2 groups of nucleic acids and proteins such as hemoglobin (17). In our study, we measured MDA concentrations after a deconjugation process in DBS samples consisting of both intracellular and extracellular fractions. Therefore, tMDA in DBS should be considered as a measure of the total lipid peroxidation in systemic circulation. Compared with traditional extracellular measures of free MDA in the plasma or serum, tMDA in DBS is potentially a more reliable and biological plausible indicator of systemic oxidation. Firstly, the likelihood of detecting tMDA in field samples is higher due to tMDA's higher level as compared to free MDA. Research has previously found that the concentration ratio of total to free MDA is 5.7 in human serum (19); this is expected to be even higher in DBS that is rich in DNA and proteins. Secondly, free MDA has been shown to have low stability in biospecimens due to its reactivity with nucleic acids and proteins (20), but this is not a concern for tMDA. Finally, MDA is not only a product of lipid peroxidation, but also a toxic molecule that directly bonds to DNA and proteins and impairs their normal function (19). Given the small fraction of extracellular DNA/protein in systemic circulation, tMDA in DBS may provide a better estimate of the total amount of essential biological molecules that are impaired by oxidative modification.

Tobacco smoke has been shown to consist of various redox active chemicals, such as free radicals, aldehydes, heavy metals, and polycyclic aromatic hydrocarbons (21). Previous studies in humans have associated exposures to secondhand smoke with the levels of free MDA in the blood and urine (22,23). Additionally, the level of total MDA in the extracellular blood fraction (e.g., serum) was associated with exposures to secondhand smoke in humans as well (24,25). In our study, we observed significantly higher levels of total MDA in DBS consisting of both intracellular and extracellular blood protein in participants exposed to secondhand smoke (*Table 2*). Taken together, these results suggested that exposures to secondhand smoke induce oxidative stress and increase the formation of MDA adducts. Remarkably, the adducts of MDA with DNA and proteins have been shown to initiate several pathological conditions in the lung, which were suggested as important mechanisms by which tobacco smoke caused adverse cardiopulmonary effects (26).

The tMDA levels in DBS were positively associated with the incidence of chest tightness and breath difficulty, supporting its usage as an early biomarker of cardiovascular health risk. Of note, chest tightness can be caused by many health conditions, such as heart attack, asthma, or hypertension (27-29). In our study, there is a high incidence of chest tightness and breath difficulty among the study participants (*Table 2*), with a greater proportion of participants having medical histories of high blood pressure as compared with asthma and cardiovascular diseases (*Table 1*). Thus, we inferred that high blood pressure might be the culprit underlying the associations between MDA and chest tightness, which is biological plausible since increased oxidative stress has been shown to promote high blood pressure through modulating endothelial function, vascular tone, and cardiac function, to cause cell and tissue damages, and to reduce the bioavailability of vasodilator nitric oxide (30).

Despite the lack of associations between CRP and worsen health outcomes, the average plasma CRP level in rural Senegalese women (7.18 μg/mL), derived from the relationship between CRP levels in plasma and cDBS in the cross-validation study (*Figure 1E*), was greater than 3 μg/mL, implying a high risk of cardiovascular diseases (31). In addition, we found that the CRP level in studied population was higher than that of many other populations worldwide (Figure S4). A likely cause is that all study participants were routinely exposed to severe household air pollution from solid fuel use. Of note, previous studies have documented elevated CRP levels in women (32) and in people with low household incomes (33), which may also contribute to the high CRP level in our Senegal participants. It should be noted that in this study we only assessed limited numbers of cardiopulmonary symptoms, it is to be addressed by future studies to what extent such a high level of CRP would impair the cardiovascular health of the studied population.

Strengths and limitations

A major strength of our study is its combination of cross- and field-validation studies to explore the usefulness of tMDA in DBS as a more convenient (than venous blood) biomarker of cardiopulmonary health risk. Our findings are promising and warrant future studies in multiple populations and with more accurate and detailed assessments of cardiopulmonary outcomes. Considering its exploratory nature, the present study has limitations including the following. First, the cross-specimen comparisons were based on a small number of volunteers (n=9). Nevertheless, previous studies with larger sample sizes have already demonstrated strong correlations of CRP levels in plasma, serum, cDBS, and vDBS (12,13). Out study is the first to observe high correlation coefficients for tMDA between cDBS and vDBS, a surrogate of whole venous blood. The correlation coefficients were comparable for tMDA and CRP (*Figure 1*), supporting the use of tMDA in DBS as a biomarker of systemic oxidation. Second, the associations between tMDA levels and cardiopulmonary outcomes and risk factors could be affected by unmeasured potential confounders (e.g., acute viral and bacterial infection). The effect of time-varying factors could not be completely eliminated in our longitudinal analysis of repeated measures in which participants served as their own controls. Third, the assessment of adverse cardiopulmonary effects was based on questionnaires, which may be subject to recall bias and result in misclassification. However, the misclassification error is likely to be random which would bias the association to null. Therefore, real associations may be stronger. Last, we did not collect plasma or vDBS samples in the field study in Senegal. Hence, it is unclear whether tMDA levels in cDBS samples would exhibit stronger associations with health outcomes than those in the plasma, and this issue warrants future study.

In summary, we found a strong correlation for tMDA and CRP in cDBS and vDBS samples. Given that CRP and MDA are well established biomarkers of systemic inflammation and lipid peroxidation, respectively, DBS specimens can serve as a convenient alternative to venous blood samples when used to analyze these biomarkers. Moreover, we found high CRP levels in a cohort of 441 rural Senegalese women exposed to high background levels of household air pollution. Higher tMDA levels in DBS samples were significantly associated with chest tightness incidence and secondhand smoke exposure. Taken together, our exploratory findings indicate that measurement of tMDA in DBS provides a promising lipids-damage-based biomarker for cardiopulmonary health risks.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/jtd-21-604). The series "Air Pollution Section" was commissioned by the editorial office without any funding or sponsorship. JZ serves as the unpaid Guest Editor of the series and an unpaid editorial board member of *Journal of Thoracic Disease*. The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study consisted of cross-validation and field-validation components, both of which conformed to the provisions of the Declaration of Helsinki (as revised in 2013) and were approved by the institutional review board of Duke University (Protocols 2018-0061 and 2020-0297). All participants gave their informed consent to participate prior to measurements.

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