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Lung clearance index may detect early peripheral lung disease in sickle cell anemia

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ABSTRACT

Rationale. Chronic lung injury is common in sickle cell anemia (SCA) and worsens outcomes. Sensitive lung function tests might predict reversible disease that might benefit from therapeutic interventions.

Objective. To evaluate whether Lung Clearance Index (LCI), S_{acin} & $S_{cond,}$ measuring global, intracinar & conductive ventilation inhomogeneity respectively, are more frequently abnormal than lung volumes in young people with SCA.

Methods. Nitrogen multiple breath washout, spirometry and body plethysmography were cross-sectionally evaluated at steady state in subjects with SCA (hemoglobin SS) and healthy controls aged 8-21 years from London, UK.

Results. 35 patients (51% boys; mean±SD 16.4±3.5 years) and 31 controls (48% boys; 16.2±3.2 years) were tested. There were significant differences between the study and control group in mean LCI (mean difference 0.42 units, 95%CI 0.22 to 0.63, p = 0.0001), S_{acin} (mean difference 0.014 units, 95%CI 0.001 to 0.026, p = 0.04), FEV_1 (mean difference -0.79 z-scores, 95%CI -1.28 to -0.30, p = 0.002), FVC (mean difference -0.80 z-scores, 95%CI -1.28 to -0.31) and TLC (mean difference -0.79 z-scores, 95%CI -1.25 to -0.29), but not in S_{cond} and FEV_1/FVC ratio. While 29% (10/35) of patients had LCI >95th percentile of controls, 23% (8/35) had abnormal FEV_1 (<5th of the reference population).

Conclusion. Lung clearance index detected slightly more abnormalities than lung volumes in young people with SCA. Significant differences with controls in LCI and S_{acin} but not in S_{cond} and FEV_1/FVC ratio suggest that the lung function changes were most likely due to patchy peripheral lung disease.

INTRODUCTION

Respiratory morbidity is common in sickle cell anemia (SCA) and is one of the leading causes of mortality in people with this condition.[1] Mechanisms of pulmonary damage in SCA involve parenchyma, airways and/or pulmonary vasculature, resulting in both acute and chronic complications.[2] Most children with SCA have lung function within the limits of normal.[3] However, these subjects generally experience a decline of lung function with increasing age,[4–6] likely reflecting the progression of end-organ lung damage over time.[7] While obstructive physiology is detected in 10-25% of school-age children with SCA[5,8,9] restrictive abnormalities become more prevalent towards adolescence[5,10] and have been reported in over 70% of adults with this condition.[6]

In an era of emerging disease modifying therapies for SCA,[11] the early detection of lung function abnormalities might offer a window of opportunity for interventions aimed to prevent chronic pulmonary deterioration in patients with this condition. The Lung Clearance Index (LCI) from Multiple Breath Washout (MBW) reflects global inhomogeneity of ventilation distribution in the lungs and is a sensitive marker of early lung disease in young children with cystic fibrosis (CF),[12] where raised LCI mainly depends on the presence of patchy airway disease.

Among MBW indices, in addition to LCI, the phase III slope derived indices S_{cond} & S_{acin} may help to clarify the mechanisms of gas mixing inefficiency and, potentially, to localise pathology along the respiratory tract.[13] In particular, S_{cond} is presumed to measure convection-dependent ventilation inhomogeneity (CDI) arising within the conductive airways; S_{acin} is presumed to measure diffusion-convection interaction dependent ventilation inhomogeneity (DCDI), deriving from the interaction between the two components at the entry of the acinar region.[14] These indices have not been previously evaluated in people with SCA.

Individuals with Hemoglobin (Hb) SS or Sß^0 disease might have ventilation distribution inhomogeneity, due to the variable combination of intracinar changes (*i.e*. interstitial fibrosis and lobar volume loss[15,16]) and conductive airway abnormalities (*i.e.* chronic airways inflammation and remodelling,[17] compression of small airways by congested peripheral pulmonary vessels[18]). Machogu et al found normal LCI values in young patients with sickle cell disease.[13] However, that cohort included also patients with compound heterozygosity for Hb S and C, who generally have a milder course of disease than those with HbSS or HbSß^0. Increased ventilation inhomogeneity at single breath washout (SBW) was previously reported in approximately 60% of adults with SCA not acutely ill.[14]

We hypothesized that, similarly to CF despite different pathophysiology mechanisms, MBW could detect early SCA-related chronic lung disease. In this cross-sectional study, we aimed to evaluate whether that MBW is more sensitive than spirometry and plethysmography at distinguishing between disease and health when used in young people with SCA evaluated at steady state[19] and healthy controls.

METHODS

This cross-sectional study enrolled individuals with SCA (HbSS) of black-African origin (African or Caribbean), aged 8–21 years, alongside healthy controls matched for ethnic group and for ±1 year of age. Assessments were performed at the Lung Function Laboratory of the Great Ormond Street Hospital for Children NHS Trust, London between October 2019 and March 2020.

After ethics committee approval, patients with SCA and controls were recruited as part of follow-up for the Sleep Asthma cohort [20](NRES London – West London & GTAC - 15/LO/0347) and a prospective study of sleep (UCL REC 14475/001) and among friends

and relatives of participant in these studies (more details in the online supplement - OLS). Both these cohorts had recruited unselected SCA patients. Written consent was obtained from participants aged 16 years and older or from the parents of younger participants, who provided written assent. Exclusion criteria were the occurence of SCA-related acute events (eg, pain crises) in the last month, smoking, premature birth, the presence of respiratory symptoms or feeling unwell on the test day and the inability to perform qualitatively acceptable lung function tests (only LCI for MBW). Controls with a history of asthma (family reported doctor's diagnosis) were also excluded, as normative values for MBW were needed.

Caregivers and participants were interviewed via a questionnaire investigating different aspects, including medical history (details in the OLS). The information regarding current medication, previous stroke and acute chest syndrome (ACS) was also verified by medical records (ACS, defined as fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on a chest X-ray).[21]

Assessments

Anthropometric parameters were measured and converted to z-scores.[22] Nitrogen MBW (Exhalyzer®D; Eco Medics AG, Switzerland), body plethysmography and spirometry (Jaeger MasterScreen®; Viasys Healthcare, Germany, running V3.0.3 Sentrysuite Software Version) were performed in this order on the same day according to European Respiratory Society/American Thoracic Society (ERS/ATS) standards[23–25] adapted for children for participants <14 years of age for spirometry and plethysmography.[26,27] Nitrogen MBW was originally performed using Spiroware Version 3.1.6 software. However, recently published data demonstrated a cross-talk error between the CO_2 and O_2 analysers used in the Exhalyzer® D to derive N_2 concentration, which results in N_2 concentration measurement error and overestimation of FRC and LCI by 10-15%.[28,29] A

correction algorithm has been incorporated in the recently released Spiroware v3.3.1,[28] therefore all data were re-analyzed with this software version.

Multiple Breath Washout outcomes were calculated as the average of at least 2 (preferably 3) acceptable washouts for each subject (*i.e.*, functional residual capacity [FRC] variation among trials less than 25%, stable breathing pattern, no leaks or marked volume drift or end tidal CO_2 concentration drift out of the range of 4-6% during the washout).[24] Lung Clearance Index represents the number of lung air turnovers (TO, 1 TO = cumulative expired volume that equals the FRC) needed to washout the lungs down to a 1/40 of the initial N_2 concentration. Normative values for LCI in Caucasian children have been published[30] but could not be used due to the different ethnicity and different software version compare with the current study. Lung clearance index results were reported as absolute values as they do not have a significant relationship with height or age in children older than 6 years.[31]

To compute S_{cond} & S_{acin}, alveolar phase III slope values on the spirogram of each breath were measured at 55-95% of the expired volume, divided by mean gas concentration over the phase III interval and multiplied by tidal volume in litres (S_{nIII}).[32] S_{cond} quantifies the S_{nIII} increase between 1.5 and 6.0 TO of the washout that, in the presence of abnormal ventilation distribution, arises because successive breaths preferentially deplete the best ventilated lung regions first, exaggerating differences in N_2 concentration measured at mouth.[13] S_{acin} is calculated from the S_{nIII} of the first breath minus CDI contribution.[24] Breaths without a clear linear phase III portion of the expirogram, or irregular expiration and oscillation, or with a phase III volume <50% or >75% of the total expired tidal volume, were excluded from S_{nIII} analysis.[24] S_{acin} & S_{cond} were calculated, respectively, only for trials with a first breath of adequate quality and with at least 2/3 of S_{nIII} values left after quality control.[24] The upper limit of normal (ULN) for LCI, S_{cond} & S_{acin} was established at

95th percentile (pc) of the control population. Further details on MBW and S_{nIII} analysis are provided in the OLS.

Spirometry values were converted to z-scores according to the Global Lung Function Initiative 2012 (GLI-2012) prediction equations for African–Americans.[33] Static lung volumes z-scores were derived according to the GLI equations for static lung volumes in individuals of European ancestry,[34] with a black African ethnic group adjustment of 12% for TLC and 7% for FRC & RV.[35] The lower limit of normal (LLN) for dynamic and static lung volumes corresponded to -1.64 z-scores of predicted (5° pc).

Lung volumes patterns were categorized according to spirometry and TLC results using the algorithm proposed in figure 1.[9]

Power of study & statistical analysis

Based on the results of an interim analysis performed on the first 20 patients and 20 controls tested, the inclusion of at least 30 patients and 30 controls would provide 90% power at the 5% significance level (two tails) to detect differences in the mean LCI of 0.7 (hypothesizing a mean±SD LCI of 7.6±0.9 in the study group and 7.0±0.4 in controls). Normality of distribution was assessed by the *Shapiro-Wilk* test. Group comparisons were performed using unpaired *t*-test, χ^2 test or *Fisher's* exact test as appropriate. The relationship between LCI & spirometry outcomes was evaluated through Pearson correlation. The relationship between variables of interest (i.e. sex, age, current asthma, current hydroxyurea therapy, history of ACS) and MBW outcomes reflecting global ventilation inhomogeneity (LCI) and regional ventilation inhomogeneity (S_{acin} & S_{cond}) was evaluated in patients with SCA through multivariable linear regression models. Validity of regression assumptions was assessed through the analysis of residuals and heteroscedasticity of variance (*Cameron and Trivedi's* decomposition of information matrix test). To meet linear regression assumptions, outcome variables were converted to their reciprocals (1/outcome variable).

A *p* value <0.05 was considered statistically significant. Analyses were performed using STATA V.14 software, and graphs were drawn with GraphPad Prism V.8.4.2.

RESULTS

General characteristics of the study population

A total of 39 children and young people with SCA and 39 healthy controls were initially recruited. All patients were homozygous for the HbS mutation, as confirmed by hemoglobin, DNA and family studies, as appropriate. After exclusions (figure 2), 35 patients with HbSS (51% male, mean±SD 16.4±3.5 years) and 31 controls (48% male, mean±SD 16.2±3.2 years) were retained in the final analysis.

The majority of the participants (74 out of 78, 95%) had sub-Saharan African ancestry, except for 2 patients and 2 controls of African-Caribbean origin. All the patients with SCA were followed at tertiary care centres in London and were receiving similar standards of care according to current UK guidelines for this condition.[36]

Individuals with SCA were significantly shorter and thinner than healthy controls (table 1).

Seventeen out of 35 patients (49%) with SCA had experienced at least 1 episode of ACS in the past, for a total of 28 episodes recorded (range 1-4 episodes for patient).

General characteristics of the study population are presented in table 1.

Table 1. Characteristics of patients with sickle cell anemia (SCA) and healthy controls aged 8-21 years from London, UK.

	SCA group n. 35	**Control n. 31**	**Mean diff. (95% CI) *P***
Boys	18 (51%)	15 (48%)	
Age, yr	16.4 (3.5)	16.2 (3.2)	0.2 (-1.5, 1.8) *P* = 0.8
Height z-score†	-0.44 (1.22)	0.35 (1.30)	-0.79 (-1.50, -0.09) *P* = 0.02

BMI z-score†	-0.22 (0.95)	0.92 (1.15)	-1.14 (-1.73, -0.54) *P* = 0.0003
	n (%)	**n (%)**	***P***
Urban residency	33 (94%)	30 (96%)	0.5
Secondhand smoke	0	3 (10%)	0.09
Current asthma#	4 (11%)	-	-
Current hydroxyurea therapy	13 (37%)	-	-
Current chronic transfusion therapy	1 (3%)		
At least 1 VOE in the last 12 months¶	14 (40%)	-	-
Previous ACS§	17 (49%)	-	-

Abbreviations: BMI, body mass index; ACS, acute chest syndrome; VOE, vaso-occlusive pain episode; SCA, sickle cell anemia
Values presented as mean (SD) in the upper part of the table and as frequencies in the lower part
†Data for Height & BMI z-score available in 27 patients and 24 controls up to 19 years of age as anthropometry z-scores derived from WHO growth reference cover only individuals aged 5-19 years.[22]
#Based on the answer to the written question "Has your child had wheezing or whistling in the chest in the past 12 months?" or current prescription of anti-asthma medication.
¶Acute pain lasting at least 4 hours with no explanation other than vaso-occlusion, requiring urgent medical attention
§Fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on a chest X-ray.[21]

Of 4 patients (11%) with SCA and current asthma, only one had known allergic asthma and two were on low-dose prophylactic inhaled corticosteroids at the time of testing.[37]

Lung function in subjects with SCA & controls

Test failure rates in the study group were 5% (2/37) for body plethysmography (9% in controls), nil for spirometry, 3% (1/37) for LCI (6% in controls), 6% (2/37) for S_{acin} (nil in controls) and 8% (3/37) for S_{cond} (9% in controls).

Lung clearance index, S_{acin} & S_{cond} were calculated as the mean of 3 N_2MBW trials in all but 2 patients with SCA for S_{acin} and 1 for S_{cond}, who had these indices derived from 2 acceptable trials. These 3 patients had normal S_{acin} & S_{cond} values. Mean±SD tidal volume per kilogram ratio at N_2MBW was comparable in the study and control group (10.6±2.7 $mL \cdot kg^{-1}$ vs 9.8±1.9 $mL \cdot kg^{-1}$, $P = 0.15$).

The 95th pc of the control population was 6.67 for LCI, 0.084 for S_{acin} and 0.038 for S_{cond}.

Patients with SCA had FEV_1, FVC and TLC reduced by approximately 0.8 z-score (around 10% of predicted) compared with healthy controls but there were no significant differences in mean FEV_1/FVC ratio (table 2, figure 3).

Seventy-five percent (26/35) of the patients with SCA had normal lung volumes (as classified in figure 1), 14% (5/35) had a restrictive pattern, 3% (1/35) had an unspecific pattern (low FVC, normal FEV_1/FVC & TLC), 6% (2/35) an obstructive pattern and 3% (1/35) a mixed one.

Lung clearance index and S_{acin} were significantly higher (more abnormal) in the SCA group than in controls, whereas S_{cond} values were comparable in the two groups (table 2, figure 3).

Table 2. Lung function outcomes in black African and black African-Caribbean individuals with sickle cell anemia and healthy controls of 8-21 years of age.

	SCA group n. 35	**Control n. 31**	**Mean Diff (95% CI), *P***
FEV_1 z-score	-0.78 (1.17)	0.01 (0.78)	-0.79 (-1.28 to -0.30), 0.002
FVC z-score	-0.54 (1.19)	0.26 (0.73)	-0.80 (-1.28 to -0.31), 0.002
FEV_1/FVC z-score	-0.57 (0.84)	-0.47 (0.91)	-0.10 (-0.53 to 0.32), 0.6
TLC z-score	-0.68 (1.10)	0.08 (0.79)	-0.76 (-1.25 to -0.29), 0.002
FRC z-score	-0.47 (0.77)	-0.19 (0.88)	-0.28 (-0.70 to 0.12), 0.16
RV z-score	-0.10 (0.48)	-0.19 (0.88)	-0.09 (-0.23 to 0.4), 0.4

	SCA group n. 35	Control n. 31	Mean Diff (95% CI), *P*
RV/TLC%	24 (4)	23 (5)	1 (-1 to 3), 0.5
LCI	6..53 (0.49)	6.11 (0.30)	0.42 (0.22 to 0.63), 0.0001
S_{acin}[#]	0.067 (0.028)	0.053 (0.020)	0.014 (0.001 to 0.026) 0.03
S_{cond} [§]	0.021 (0.012)	0.024 (0.013)	-0.003 (-0.009 to 0.003) 0.3

Definition of abbreviations = LCI, Lung clearance index; SCA, Sickle cell anemia

Results are presented as mean (SD), unless otherwise specified.

Spirometry z-scores based on Global Lung Function Initiative 2012 equations for African Americans.[33]

Static lung volumes z-scores based on Global Lung Function Initiative equations for static lung volumes[34] with a black African ethnic group adjustment of 12% for TLC and 7% for FRC & RV.[35]

[#]Data available in 34 individuals with SCA and 31 controls

[§]Data available in 33 individuals with SCA and 30 controls

The LCI, S_{acin} & S_{cond} were abnormal (>95th centile for controls), respectively, in 29% (10/35), 18% (6/34) and 6% (2/33) of the study group, whereas FEV_1, FVC and TLC were abnormal (<-1.64 z-score), respectively, in 23% (8/35), 11% (4/35) and 17% (6/35) of the patients (figure 3).

Of 10 patients with SCA and abnormal LCI, 5 subjects had lung volumes within the limits of normal, 3 had a restrictive or unspecific pattern, 1 had a mixed pattern and 1 had an obstructive pattern.

In the SCA group, there was a moderate negative correlation between LCI and FEV_1 z-score (r = −0.45, P = 0.007), FVC z-score (r = −0.45, P = 0.006) and TLC z-score (r = -0.49, P = 0.03) but no correlation between LCI and FEV_1/FVC z-score (r = -0.07, P = 0.4) (figure 4).

On multivariable regression analysis, there were no significant associations between the predictors (sex, age, current asthma, current hydroxyurea therapy, history of ACS) and

LCI, whereas a history of ACS was associated with more pathological S_{acin} values (B for $1/S_{acin}$ = -5.6, 95% CI **(-9.7 to -1.53;**, *P* = 0.009) (table S1).

DISCUSSION

This cross-sectional study showed increased ventilation inhomogeneity in almost 30% of young people with SCA, with LCI being slightly more frequently abnormal than lung volumes. There were significant differences between individuals with SCA and controls in FEV_1, FVC, TLC, LCI and S_{acin} but not in FEV_1/FVC and S_{cond}, suggesting that gas mixing abnormalities were mainly caused by patchy parenchymal or peripheral lung disease, rather than by obstructive disease.

Lung clearance index abnormalities in patients with SCA were milder (highest value = 8.09) than reported in school-age CF patients.[38] These difference are most likely due to the different mechanisms driving gas mixing abnormalities in the two conditions. More specifically, MBW measures differences in specific ventilation between parallel lung units, which typically occur in the presence of a patchy distribution of pathology. These differences can be the result of patchy airway disease, as in early CF lung disease[39], but can also result from patchy changes in lung compliance, or by disruptions of peripheral lung architecture. A patchy distribution of lung lesions has been reported in the majority of adult patients with SCA at steady-state, with chest CT showing scattered areas of reticular pattern, ground glass opacification, linear bands and lobar volume loss.[15,16]. Moreover, when comparing N_2MBW LCI results of our cohort with those of previous studies performed with older versions of the Exhalyzed®D Spiroware software, it should be kept in mind that the correction of the cross-talk error in the new v3.3.1 may result in reductions of LCI values by 10-15% compared with v3.1.6, with larger effect expected for higher LCI values.[28] In our study, re-analysis of data with v3.3.1 resulted in a significant reduction of

average LCI by 14% in SCA patients (from 7.61±0.71 to 6.53±0.49, p <0.0001) and by 11% in controls (from 6.87±0.39 to 6.11±0.30, p <0.0001), whereas S_{acin} values were not affected and S_{cond} values were only marginally affected (table S2, OLS).

In comparison with healthy controls, in our SCA cohort we saw a restrictive physiology, raised LCI and S_{acin}, but normal FEV_1/FVC and S_{cond}. This pattern suggests that the ventilation inhomogeneity was arising by the DCDI mechanism, and hence probably represents disease of the most peripheral intracinar airways or of the lung parenchyma. From a pathophysiology perspective, changes in lung periphery might be related to recurrent episodes of vaso-occlusion and hemolysis in the pulmonary microcirculation, triggering chronic inflammation and ischemia-reperfusion injury, which in turn may lead to structural changes of lung architecture and remodelling of pulmonary vasculature.[40] Experimental studies have revealed some of the complex interactions between leucocytes (neutrophils, monocytes), erythrocytes, platelets, endothelium, adhesion molecules and inflammatory mediators that might be involved in this process,[17] while evidence from epidemiology studies supports the hypothesis of chronic end-organ lung damage in subjects with SCA.[7]

A history of ACS was associated with higher S_{acin} values at MBW. Several factors can be involved in the pathogenesis of ACS, including infection and pulmonary fat embolism, as well as sickling and vaso-occlusion in the pulmonary microcirculation leading to pulmonary infarction.[41,42] These mechanisms can contribute to determining chronic changes of the peripheral lung regions involved (*i.e.* interstitial fibrosis) that might affect DCDI and, therefore, S_{acin}.

Lopes *et al* previously reported increased ventilation inhomogeneity at nitrogen SBW (*i.e.* increased steepness of the expirogram-derived phase III slope -S_{III}-) in over half of adult patients with SCA without acute symptoms. Assuming that DCDI is the main contributor to SBW S_{III} slope,[43] our findings are consistent with those of Lopes *et al*.[44]

Compared to the SBW technique used in that study, which requires a vital capacity manoeuver performed at a low constant flow of approximately 0.5 litre/s, the MBW is easier to perform reliably in children.[24] Moreover, the LCI has the advantage of being less influenced by gravitational inter-regional differences in gas distribution during the inspiratory phase compared with the S_{III} from SBW.[14]

More recently, Machogu *et al* found normal LCI values in a cohort of African-American children and adolescents with sickle cell disease, as compared with healthy controls.[45] Our findings are difficult to compare with that study that included also individuals with HbSC (20% of the cohort), who usually have a milder course of disease than those with SCA. While almost half patients in our cohort had at least one previous episode of ACS (17/35, 49%), consistent with the reported epidemiology in individuals with Hb SS and Sß^0,[46] the frequency of ACS episodes in the study by Machogu *et al* was much lower, with a total of 11 episodes in 35 patients, 6 in the same subject.[45] Differences in the study populations were likely to be reflected not only in diverging MBW results but also in markedly better spirometry outcomes (mean±SD FEV_1 score -0.22±1.09; mean±SD FVC z-score 0.05±1.04)[33] in the cohort of Machogu *et al*, as compared with the present one (mean±SD FEV_1 score -0.78±1.17; mean±SD FVC z-score -0.54±1.19) and with other recent pediatric cohorts of patients with SCA.[5,8,9,47]

S_{cond} values in patients with SCA did not differ from those of healthy controls. S_{cond} measures convective gas mixing in the conductive airways proximal to the acinar region[14] and tends to be abnormal in in obstructive lung diseases.[48–50] Young people with SCA are known to have high rates of airway hyperresponsiveness[51] and wheezing[20] that can be associated with SCA-related chronic airway inflammation and remodelling.[17] Moreover, an obstructive physiology is the most frequent pathological lung function pattern reported in pediatric patients with this condition living in high-income countries.[3] In our SCA cohort, an obstructive or mixed lung volumes pattern was

detected in 3 out of 35 patients, 2 with LCI >95° pc of controls (figure 4). In these subjects, the increased ventilation inhomogeneity was more likely to be due to conductive airway impairment rather than to peripheral lung damage, showing another aspect of the spectrum of manifestations of SCA-related chronic lung disease

Strengths and limitations

The main strength of this study is the use of MBW to assess respiratory impairment in patients with SCA, which has so far received relatively little attention. The integration of results from different tests allowed a comprehensive evaluation of lung function. Among the MBW indices, the calculation of S_{acin} & S_{cond}, in addition to more commonly used LCI, provided insight into the possible localization of pathology along the airway tree. Moreover, this is among the first studies presenting S_{acin} & S_{cond} values obtained by Spiroware 3.3.1 and demonstrating that regional ventilation inhomogeneity indices are not significantly affected by the correction of the error in the nitrogen concentration calculation of the Exhalyzer®D. The presence of a control group matched for ethnicity and age (± 1 year) was essential for the correct interpretation of MBW and body plethysmography findings, in the absence of validated references values for people of African ancestry.

The study also has limitations. Most importantly, the absence of simultaneous lung imaging assessment prevents us from relating the MBW abnormalities to specific structural alterations, which can only be speculated upon. Given the absence of published normative values for N_2MBW in children and adolescents of black African origin, we could only establish the ULN for MBW parameters according to the distribution of values in our control population but we cannot exclude that ULN values derived from a large reference population would have been different, affecting the results. Moreover, the cross-sectional design does not allow us to draw conclusions regarding the clinical and prognostic meaning of LCI alterations in patients with SCA. It should also be taken into account that a

minimal clinically important difference for LCI values has not been established so far, therefore we cannot be certain that the difference found in mean LCI between patients and controls (0.42, 95% CI 0.22 to 0.63), albeit highly statistically significant (*P* 0.0001), corresponds to relevant differences in terms of respiratory pathophysiology. Differences in S_{acin} values between SCA patients and controls were influenced by the presence of a patient with very high S_{acin} (0.170 units), without whom statistical significance would be missed (*p* would change from 0.03 to 0.06). However, a trend towards higher S_{acin} values in SCA patients than in controls would be still evident also without this subject (figure 3, more details in OLS). In view of the above, both the finding of greater ventilation inhomogeneity in SCA patients and the mechanisms driving this will need further confirmation in future studies. The absence of prospective longitudinal data and the small sample size also limited the evaluation of risk factors for pathological MBW results. In particular, the study was not powered for multivariable regression analysis. Furthermore, the use of S_{cond} and S_{acin} as proxies for regional ventilation inhomogeneity is based on assumptions that have not been validated in the pediatric population and, therefore, the interpretation of results cannot be certain. Finally, the study is only representative of subjects with SCA followed at tertiary care centres, who, however, represent the great majority of patients living in high-income countries.

Future directions

Future studies should further investigate whether ventilation inhomogeneity is frequently abnormal in patients with SCA and whether the LCI is useful to track early respiratory disease in young people with this condition. To explore this hypothesis, a prospective longitudinal study of adequate power and involving monitoring of lung function, chest imaging (*i.e.* chest CT, functional MRI of the lung), clinical events and adjustment for confounders (including therapies received) would be required. If an abnormal LCI were

proven to correlate with structural lung abnormalities, more rapid decline of lung function or a worse clinical course, the use of MBW could be considered in the routine respiratory of patients with SCA (as detection of early lung disease would offer a window of opportunity for therapeutic interventions) and/or as possible endpoint in clinical trials evaluating disease-modifying drugs in this group.

Conclusion

This study showed that the MBW is more frequently abnormal than spirometry and body plethysmography in young patients with SCA. We found mildly increased ventilation inhomogeneity in people with SCA, which appears to result in most cases from peripheral or parenchymal lung disease, rather than obstructive disease.

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Figure 1. Lung volumes pattern classification according to spirometry and total lung capacity values in individuals with sickle cell anemia. Modified from *Cohen RT. et al*[9].
Abbreviations: FEV_1 = Forced expired volume in the 1st second; FVC = Forced vital capacity; TLC = Total Lung Capacity; SCA = Sickle cell anemia;
Low = <-1.64 z-scores predicted according to the Global Lung Function Initiative reference values for spirometry[33] ("Black" equation) and static lung volumes[34] with 12% ethnic group correction for TLC.[35]

Figure 2. Study population.
Abbreviations: SCA = sickle cell anemia.

Figure 3. Comparison of lung function outcomes in 35 individuals with sickle cell anemia (SCA, black dots) and 31 healthy controls (white dots) aged 8-21 years. The straight bars indicate the mean values with 95% confidence interval in each group. The dashed lines indicate the limits of normal, corresponding to the 5th percentile of the reference population[33,34] for FEV_1, FVC, FEV_1/FVC and TLC (12% ethnic group correction for TLC[35]) and 95th percentile of the controls for LCI.
Abbreviations**:** SCA = Sickle cell anemia; $zFEV_1$ = Z-score forced expired volume in the 1st second; zFVC = Z-score forced vital capacity; zTLC = Z-score total lung capacity; LCI = Lung clearance index; LLN = Lower limit of normal; ULN = Upper limit of normal

Figure 4. LCI values plotted against FEV_1 z-scores (A), FVC z-scores (B), FEV_1/FVC z-scores (C) and TLC z-scores (D) in 35 individuals with sickle cell anemia (black dots) and 31 healthy controls (white dots) aged 8-21 years. The dashed lines indicate the limits of normal, corresponding to the 5th percentile of the reference population for FEV_1, FVC & FEV_1/FVC z-scores[33] and TLC z-scores[34] (12% ethnic group correction for TLC[35]) and to the 95th percentile of the control group for LCI.
Abbreviations: LCI = Lung clearance index; FVC = Forced Volume Capacity; FEV_1 = Forced Expired Volume in the 1st second; TLC = Z-score total lung capacity; LLN = Lower limit of normal; ULN = Upper limit of normal

Lung clearance index may detect early peripheral lung disease in sickle cell anemia

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ABSTRACT

Rationale. Chronic lung injury is common in sickle cell anemia (SCA) and worsens outcomes. Sensitive lung function tests might predict reversible disease that might benefit from therapeutic interventions.

Objective. To evaluate whether Lung Clearance Index (LCI), S_{acin} & $S_{cond,}$ measuring global, intracinar & conductive ventilation inhomogeneity respectively, are more frequently abnormal than lung volumes in young people with SCA.

Methods. Nitrogen multiple breath washout, spirometry and body plethysmography were cross-sectionally evaluated at steady state in subjects with SCA (hemoglobin SS) and healthy controls aged 8-21 years from London, UK.

Results. 35 patients (51% boys; mean±SD 16.4±3.5 years) and 31 controls (48% boys; 16.2±3.2 years) were tested. There were significant differences between the study and control group in mean LCI (mean difference 0.42~~74~~ units, 95%CI 0.22 to 0.63, *p* = 0.0001~~0.45 to 1.02~~), S_{acin} (mean difference 0.01~~43~~ units, 95%CI ~~0~~0.001 to 0.026~~.000 to 0.0024~~, *p* = 0.04), FEV_1 (mean difference -0.79 z-scores, 95%CI -1.28 to -0.30, *p* = 0.002), FVC (mean difference -0.80 z-scores, 95%CI -1.28 to -0.31) and TLC (mean difference -0.79 z-scores, 95%CI -1.25 to -0.29), but not in S_{cond} and FEV_1/FVC ratio. While ~~43~~29% (10~~5~~/35) of patients had LCI >95th percentile of controls, 23% (8/35) had abnormal FEV_1 (<5th of the reference population).

Conclusion. Lung clearance index detected slightly more abnormalities than lung volumes in young people with SCA. Significant differences with controls in LCI and S_{acin} but not in S_{cond} and FEV_1/FVC ratio suggest that the lung function changes were most likely due to patchy peripheral lung disease.

INTRODUCTION

Respiratory morbidity is common in sickle cell anemia (SCA) and is one of the leading causes of mortality in people with this condition.[1] Mechanisms of pulmonary damage in SCA involve parenchyma, airways and/or pulmonary vasculature, resulting in both acute and chronic complications.[2] Most children with SCA have lung function within the limits of normal.[3] However, these subjects generally experience a decline of lung function with increasing age,[4–6] likely reflecting the progression of end-organ lung damage over time.[7] While obstructive physiology is detected in 10-25% of school-age children with SCA[5,8,9] restrictive abnormalities become more prevalent towards adolescence[5,10] and have been reported in over 70% of adults with this condition.[6]

In an era of emerging disease modifying therapies for SCA,[11] the early detection of lung function abnormalities might offer a window of opportunity for interventions aimed to prevent chronic pulmonary deterioration in patients with this condition. The Lung Clearance Index (LCI) from Multiple Breath Washout (MBW) reflects global inhomogeneity of ventilation distribution in the lungs and is a sensitive marker of early lung disease in young children with cystic fibrosis (CF),[12] where raised LCI mainly depends on the presence of patchy airway disease.

Among MBW indices, in addition to LCI, the phase III slope derived indices S_{cond} & S_{acin} may help to clarify the mechanisms of gas mixing inefficiency and, potentially, to localise pathology along the respiratory tract.[13] In particular, S_{cond} is presumed to measure convection-dependent ventilation inhomogeneity (CDI) arising within the conductive airways; S_{acin} is presumed to measure diffusion-convection interaction dependent ventilation inhomogeneity (DCDI), deriving from the interaction between the two components at the entry of the acinar region.[14] These indices have not been previously evaluated in people with SCA.

Individuals with Hemoglobin (Hb) SS or Sß^0 disease might have ventilation distribution inhomogeneity, due to the variable combination of intracinar changes (*i.e*. interstitial fibrosis and lobar volume loss[15,16]) and conductive airway abnormalities (*i.e.* chronic airways inflammation and remodelling,[17] compression of small airways by congested peripheral pulmonary vessels[18]). Machogu et al found normal LCI values in young patients with sickle cell disease.[13] However, that cohort included also patients with compound heterozygosity for Hb S and C, who generally have a milder course of disease than those with HbSS or HbSß^0. Increased ventilation inhomogeneity at single breath washout (SBW) was previously reported in approximately 60% of adults with SCA not acutely ill.[14]

We hypothesized that, similarly to CF despite different pathophysiology mechanisms, MBW could detect early SCA-related chronic lung disease ~~earlier than spirometry and body plethysmography~~. In this cross-sectional study, we aimed to evaluate whether that MBW is more sensitive than spirometry and plethysmography at distinguishing between disease and health when used in young people with SCA evaluated at steady state[19] and healthy controls.

METHODS

This cross-sectional study enrolled individuals with SCA (HbSS) of black-African origin (African or Caribbean), aged 8–21 years, alongside healthy controls matched for ethnic group and for ±1 year of age. Assessments were performed at the Lung Function Laboratory of the Great Ormond Street Hospital for Children NHS Trust, London between October 2019 and March 2020.

After ethics committee approval, patients with SCA and controls were recruited as part of follow-up for the Sleep Asthma cohort [20](NRES London – West London & GTAC -

15/LO/0347) and a prospective study of sleep (UCL REC 14475/001) and among friends and relatives of participant in these studies (more details in the online supplement - OLS). Both these cohorts had recruited unselected SCA patients. Written consent was obtained from participants aged 16 years and older or from the parents of younger participants, who provided written assent. Exclusion criteria were the occurence of SCA-related acute events (eg, pain crises) in the last month, smoking, premature birth, the presence of respiratory symptoms or feeling unwell on the test day and the inability to perform qualitatively acceptable lung function tests (only LCI for MBW). Controls with a history of asthma (family reported doctor's diagnosis) were also excluded, as normative values for MBW were needed.

Caregivers and participants were interviewed via a questionnaire investigating different aspects, including medical history (details in the OLS). The information regarding current medication, previous stroke and acute chest syndrome (ACS) was also verified by medical records (ACS, defined as fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on a chest X-ray).[21]

Assessments

Anthropometric parameters were measured and converted to z-scores.[22] Nitrogen MBW (Exhalyzer®D; Eco Medics AG, Switzerland), body plethysmography and spirometry (Jaeger MasterScreen®; Viasys Healthcare, Germany, running V3.0.3 Sentrysuite Software Version) were performed in this order on the same day according to European Respiratory Society/American Thoracic Society (ERS/ATS) standards[23–25] adapted for children for participants <14 years of age for spirometry and plethysmography.[26,27]

Nitrogen MBW was originally performed using Spiroware Version 3.1.6 software. However, recently published data demonstrated a cross-talk error between the CO_2 and O_2 analysers used in the Exhalyzer® D to derive N_2 concentration, which results in N_2

concentration measurement error and overestimation of FRC and LCI by 10-15%.[28,29] A correction algorithm has been incorporated in the recently released Spiroware v3.3.1,[28] therefore all data were re-analyzed with this software version.

Multiple Breath Washout outcomes were calculated as the average of at least 2 (preferably 3) acceptable washouts for each subject (*i.e.*, functional residual capacity [FRC] variation among trials less than 25%, stable breathing pattern, no leaks or marked volume drift or end tidal CO_2 concentration drift out of the range of 4-6% during the washout).[24] Lung Clearance Index represents the number of lung air turnovers (TO, 1 TO = cumulative expired volume that equals the FRC) needed to washout the lungs down to a 1/40 of the initial N_2 concentration. Normative values for LCI in Caucasian children ~~were~~ have been ~~recently~~ published[30] but could not be used due to the different ethnicity ~~of~~ and different software version compare with the current study. Lung clearance index results were reported as absolute values as they do not have a significant relationship with height or age in children older than 6 years.[31]

To compute S_{cond} & S_{acin}, alveolar phase III slope values on the spirogram of each breath were measured at 55-95% of the expired volume, divided by mean gas concentration over the phase III interval and multiplied by tidal volume in litres (S_{nIII}).[32] S_{cond} quantifies the S_{nIII} increase between 1.5 and 6.0 TO of the washout that, in the presence of abnormal ventilation distribution, arises because successive breaths preferentially deplete the best ventilated lung regions first, exaggerating differences in N_2 concentration measured at mouth.[13] S_{acin} is calculated from the S_{nIII} of the first breath minus CDI contribution.[24] Breaths without a clear linear phase III portion of the expirogram, or irregular expiration and oscillation, or with a phase III volume <50% or >75% of the total expired tidal volume, were excluded from S_{nIII} analysis.[24] S_{acin} & S_{cond} were calculated, respectively, only for trials with a first breath of adequate quality and with at least 2/3 of S_{nIII} values left after quality control.[24] The upper limit of normal (ULN) for LCI, S_{cond} & S_{acin} was established at

95th percentile (pc) of the control population. Further details on MBW and S_{nIII} analysis are provided in the OLS.

Spirometry values were converted to z-scores according to the Global Lung Function Initiative 2012 (GLI-2012) prediction equations for African–Americans.[33] Static lung volumes z-scores were derived according to the GLI equations for static lung volumes in individuals of European ancestry,[34] with a black African ethnic group adjustment of 12% for TLC and 7% for FRC & RV.[35] The lower limit of normal (LLN) for dynamic and static lung volumes corresponded to -1.64 z-scores of predicted (5° pc).

Lung volumes patterns were categorized according to spirometry and TLC results using the algorithm proposed in figure 1.[9]

Power of study & statistical analysis

Based on the results of an interim analysis performed on the first 20 patients and 20 controls tested, the inclusion of at least 30 patients and 30 controls would provide 90% power at the 5% significance level (two tails) to detect differences in the mean LCI of 0.7 (hypothesizing a mean±SD LCI of 7.6±0.9 in the study group and 7.0±0.4 in controls). Normality of distribution was assessed by the *Shapiro-Wilk* test. Group comparisons were performed using unpaired *t*-test, χ^2 test or *Fisher's* exact test as appropriate. The relationship between LCI & spirometry outcomes was evaluated through Pearson correlation. The relationship between variables of interest (i.e. sex, age, current asthma, current hydroxyurea therapy, history of ACS) and MBW outcomes reflecting global ventilation inhomogeneity (LCI) and regional ventilation inhomogeneity (S_{acin} & S_{cond}) was evaluated in patients with SCA through multivariable linear regression models. Validity of regression assumptions was assessed through the analysis of residuals and heteroscedasticity of variance (*Cameron and Trivedi's* decomposition of information matrix test). To meet linear regression assumptions, outcome variables were converted to their reciprocals (1/outcome variable).

A *p* value <0.05 was considered statistically significant. Analyses were performed using STATA V.14 software, and graphs were drawn with GraphPad Prism V.8.4.2.

RESULTS

General characteristics of the study population

A total of 39 children and young people with SCA and 39 healthy controls were initially recruited. All patients were homozygous for the HbS mutation, as confirmed by hemoglobin, DNA and family studies, as appropriate. After exclusions (figure 2), 35 patients with HbSS (51% male, mean±SD 16.4±3.5 years) and 31 controls (48% male, mean±SD 16.2±3.2 years) were retained in the final analysis.

The majority of the participants (74 out of 78, 95%) had sub-Saharan African ancestry, except for 2 patients and 2 controls of African-Caribbean origin. All the patients with SCA were followed at tertiary care centres in London and were receiving similar standards of care according to current UK guidelines for this condition.[36]

Individuals with SCA were significantly shorter and thinner than healthy controls (table 1).

Seventeen out of 35 patients (49%) with SCA had experienced at least 1 episode of ACS in the past, for a total of 28 episodes recorded (range 1-4 episodes for patient).

General characteristics of the study population are presented in table 1.

Table 1. Characteristics of patients with sickle cell anemia (SCA) and healthy controls aged 8-21 years from London, UK.

	SCA group n. 35	**Control n. 31**	**Mean diff. (95% CI) *P***
Boys	18 (51%)	15 (48%)	
Age, yr	16.4 (3.5)	16.2 (3.2)	0.2 (-1.5, 1.8) *P* = 0.8
Height z-score†	-0.44 (1.22)	0.35 (1.30)	-0.79 (-1.50, -0.09) *P* = 0.02

BMI z-score†	-0.22 (0.95)	0.92 (1.15)	-1.14 (-1.73, -0.54) *P* = 0.0003
	n (%)	**n (%)**	***P***
Urban residency	33 (94%)	30 (96%)	0.5
Secondhand smoke	0	3 (10%)	0.09
Current asthma#	4 (11%)	-	-
Current hydroxyurea therapy	13 (37%)	-	-
Current chronic transfusion therapy	1 (3%)		
At least 1 VOE in the last 12 months¶	14 (40%)	-	-
Previous ACS§	17 (49%)	-	-

Abbreviations: BMI, body mass index; ACS, acute chest syndrome; VOE, vaso-occlusive pain episode; SCA, sickle cell anemia
Values presented as mean (SD) in the upper part of the table and as frequencies in the lower part
†Data for Height & BMI z-score available in 27 patients and 24 controls up to 19 years of age as anthropometry z-scores derived from WHO growth reference cover only individuals aged 5-19 years.[22]
#Based on the answer to the written question "Has your child had wheezing or whistling in the chest in the past 12 months?" or current prescription of anti-asthma medication.
¶Acute pain lasting at least 4 hours with no explanation other than vaso-occlusion, requiring urgent medical attention
§Fever and/or respiratory symptoms, accompanied by a new pulmonary infiltrate on a chest X-ray.[21]

Of 4 patients (11%) with SCA and current asthma, only one had known allergic asthma and two were on low-dose prophylactic inhaled corticosteroids at the time of testing.[37]

Lung function in subjects with SCA & controls

Test failure rates in the study group were 5% (2/37) for body plethysmography (9% in controls), nil for spirometry, 3% (1/37) for LCI (6% in controls), 6% (2/37) for S_{acin} (nil in controls) and 8% (3/37) for S_{cond} (9% in controls).

Lung clearance index, S_{acin} & S_{cond} were calculated as the mean of 3 N_2MBW trials in all but 2 patients with SCA for S_{acin} and 1 for S_{cond}, who had these indices derived from 2 acceptable trials. These 3 patients had normal S_{acin} & S_{cond} values. Mean±SD tidal volume per kilogram ratio at N_2MBW was comparable in the study and control group (10.6±2.7 $mL \cdot kg^{-1}$ vs 9.8±1.9 $mL \cdot kg^{-1}$, $P = 0.15$).

The 95th pc of the control population was 67.6754 for LCI, 0.08491 for S_{acin} and 0.038 for S_{cond}.

Patients with SCA had FEV_1, FVC and TLC reduced by approximately 0.8 z-score (around 10% of predicted) compared with healthy controls but there were no significant differences in mean FEV_1/FVC ratio (table 2, figure 3).

Seventy-five percent (26/35) of the patients with SCA had normal lung volumes (as classified in figure 1), 14% (5/35) had a restrictive pattern, 3% (1/35) had an unspecific pattern (low FVC, normal FEV_1/FVC & TLC), 6% (2/35) an obstructive pattern and 3% (1/35) a mixed one.

Lung clearance index and S_{acin} were significantly higher (more abnormal) in the SCA group than in controls, whereas S_{cond} values were comparable in the two groups (table 2, figure 3).

Table 2. Lung function outcomes in black African and black African-Caribbean individuals with sickle cell anemia and healthy controls of 8-21 years of age.

	SCA group n. 35	**Control n. 31**	**Mean Diff (95% CI), *P***
FEV_1 z-score	-0.78 (1.17)	0.01 (0.78)	-0.79 (-1.28 to -0.30), 0.002
FVC z-score	-0.54 (1.19)	0.26 (0.73)	-0.80 (-1.28 to -0.31), 0.002
FEV_1/FVC z-score	-0.57 (0.84)	-0.47 (0.91)	-0.10 (-0.53 to 0.32), 0.6
TLC z-score	-0.68 (1.10)	0.08 (0.79)	-0.76 (-1.25 to -0.29), 0.002
FRC z-score	-0.47 (0.77)	-0.19 (0.88)	-0.28 (-0.70 to 0.12), 0.16

	SCA group n. 35	Control n. 31	Mean Diff (95% CI), *P*
RV z-score	-0.10 (0.48)	-0.19 (0.88)	-0.09 (-0.23 to 0.4), 0.4
RV/TLC%	24 (4)	23 (5)	1 (-1 to 3), 0.5
LCI	6.7.5361 (0.4971)	6.1187 (0.309)	0.4274 (0.2245 to 0.631.02), <0.0001
S_{acin}[#]	0.067 (0.0287)	0.0534 (0.020)	0.0143 (0.0010 to 0.0024026) 0.034
S_{cond} [§]	0.02119 (0.01209)	0.0241 (0.01308)	-0.0032 (-0.0097 to 0.0032) 0.3

Definition of abbreviations = LCI, Lung clearance index; SCA, Sickle cell anemia

Results are presented as mean (SD), unless otherwise specified.

Spirometry z-scores based on Global Lung Function Initiative 2012 equations for African Americans.[33]

Static lung volumes z-scores based on Global Lung Function Initiative equations for static lung volumes[34] with a black African ethnic group adjustment of 12% for TLC and 7% for FRC & RV.[35]

[#]Data available in 34 individuals with SCA and 31 controls

[§]Data available in 33 individuals with SCA and 30 controls

The LCI, S_{acin} & S_{cond} were abnormal (>95th centile for controls), respectively, in 2943% (1015/35), 185% (65/34) and 6% (2/33) of the study group, whereas FEV_1, FVC and TLC were abnormal (<-1.64 z-score), respectively, in 23% (8/35), 11% (4/35) and 17% (6/35) of the patients (figure 3).

Of 15 10 patients with SCA and abnormal LCI, 10 5 subjects had lung volumes within the limits of normal, 3 had a restrictive or unspecific pattern, 1 had a mixed pattern and 1 had an obstructive pattern.

In the SCA group, there was a moderate negative correlation between LCI and FEV_1 z-score (r = −0.454, P = 0.007), FVC z-score (r = −0.450, P = 0.006) and TLC z-score (r = -0.490, 95% CI -0.64, -0.08, P = 0.032) but no correlation between LCI and FEV_1/FVC z-score (r = -0.0718, 95% CI -0.48 to 0.16, P = 0.43) (figure 4).

On multivariable regression analysis, there were no significant associations between the predictors (sex, age, current asthma, current hydroxyurea therapy, history of ACS) and

LCI, whereas a history of ACS was associated with more pathological S_{acin} values (B for $1/S_{acin}$ = -5.65, 95% CI (-9.78 to -1.53.28;, *P* = 0.009) (table S1).

DISCUSSION

This cross-sectional study showed increased ventilation inhomogeneity in ~~more than 40%~~almost 30% of young people with SCA, with LCI being slightly more frequently abnormal than lung volumes. There were significant differences between individuals with SCA and controls in FEV_1, FVC, TLC, LCI and S_{acin} but not in FEV_1/FVC and S_{cond}, suggesting that gas mixing abnormalities were mainly caused by patchy parenchymal or peripheral lung disease, rather than by obstructive disease.

Lung clearance index abnormalities in patients with SCA were ~~smaller~~ milder (highest value = ~~9.51~~8.09) than reported in school-age CF patients.[38] These difference are most likely due to the different mechanisms driving gas mixing abnormalities in the two conditions. More specifically, MBW measures differences in specific ventilation between parallel lung units, which typically occur in the presence of a patchy distribution of pathology. These differences can be the result of patchy airway disease, as in early CF lung disease[39], but can also result from patchy changes in lung compliance, or by disruptions of peripheral lung architecture. A patchy distribution of lung lesions has been reported in the majority of adult patients with SCA at steady-state, with chest CT showing scattered areas of reticular pattern, ground glass opacification, linear bands and lobar volume loss.[15,16]. Moreover, when comparing N_2MBW LCI results of our cohort with those of previous studies performed with older versions of the Exhalyzed®D Spiroware software, it should be kept in mind that the correction of the cross-talk error in the new v3.3.1 may result in reductions of LCI values by 10-15% compared with v3.1.6, with larger effect expected for higher LCI values.[28] In our study, re-analysis of data with v3.3.1

resulted in a significant reduction of average LCI by 14% in SCA patients (from 7.61±0.71 to 6.53±0.49, p <0.0001) and by 11% in controls (from 6.87±0.39 to 6.11±0.30, p <0.0001), whereas S_{acin} values were not affected and S_{cond} values were only marginally affected (table S2, OLS).

In comparison with healthy controls, in our SCA cohort we saw a restrictive physiology, raised LCI and S_{acin}, but normal FEV_1/FVC and S_{cond}. This pattern suggests that the ventilation inhomogeneity was arising by the DCDI mechanism, and hence probably represents disease of the most peripheral intracinar airways or of the lung parenchyma. From a pathophysiology perspective, changes in lung periphery might be related to recurrent episodes of vaso-occlusion and hemolysis in the pulmonary microcirculation, triggering chronic inflammation and ischemia-reperfusion injury, which in turn may lead to structural changes of lung architecture and remodelling of pulmonary vasculature.[40] Experimental studies have revealed some of the complex interactions between leucocytes (neutrophils, monocytes), erythrocytes, platelets, endothelium, adhesion molecules and inflammatory mediators that might be involved in this process,[17] while evidence from epidemiology studies supports the hypothesis of chronic end-organ lung damage in subjects with SCA.[7]

A history of ACS was associated with higher S_{acin} values at MBW. Several factors can be involved in the pathogenesis of ACS, including infection and pulmonary fat embolism, as well as sickling and vaso-occlusion in the pulmonary microcirculation leading to pulmonary infarction.[41,42] These mechanisms can contribute to determining chronic changes of the peripheral lung regions involved (*i.e.* interstitial fibrosis) that might affect DCDI and, therefore, S_{acin}.

Lopes *et al* previously reported increased ventilation inhomogeneity at nitrogen SBW (*i.e.* increased steepness of the expirogram-derived phase III slope -S_{III}-) in over half of adult patients with SCA without acute symptoms. Assuming that DCDI is the main

contributor to SBW S_{III} slope,[43] our findings are consistent with those of Lopes *et al*.[44] Compared to the SBW technique used in that study, which requires a vital capacity manoeuver performed at a low constant flow of approximately 0.5 litre/s, the MBW is easier to perform reliably in children.[24] Moreover, the LCI has the advantage of being less influenced by gravitational inter-regional differences in gas distribution during the inspiratory phase compared with the S_{III} from SBW.[14]

More recently, Machogu *et al* found normal LCI values in a cohort of African-American children and adolescents with sickle cell disease, as compared with healthy controls.[45] Our findings are difficult to compare with that study that included also individuals with HbSC (20% of the cohort), who usually have a milder course of disease than those with SCA. While almost half patients in our cohort had at least one previous episode of ACS (17/35, 49%), consistentl~~y~~ with the reported epidemiology in individuals with Hb SS and Sß^0,[46] the frequency of ACS episodes in the study by Machogu *et al* was much lower, with a total of 11 episodes in 35 patients, 6 in the same subject.[45] Differences in the study populations were likely to be reflected not only in diverging MBW results but also in markedly better spirometry outcomes (mean±SD FEV_1 score -0.22±1.09; mean±SD FVC z-score 0.05±1.04)[33] in the cohort of Machogu *et al*, as compared with the present one (mean±SD FEV_1 score -0.78±1.17; mean±SD FVC z-score -0.54±1.19) and with other recent pediatric cohorts of patients with SCA.[5,8,9,47]

S_{cond} values in patients with SCA did not differ from those of healthy controls. S_{cond} measures convective gas mixing in the conductive airways proximal to the acinar region[14] and tends to be abnormal in in obstructive lung diseases.[48–50] Young people with SCA are known to have high rates of airway hyperresponsiveness[51] and wheezing[20] that can be associated with SCA-related chronic airway inflammation and remodelling.[17] Moreover, an obstructive physiology is the most frequent pathological lung function pattern reported in pediatric patients with this condition living in high-income

countries.[3] In our SCA cohort, an obstructive or mixed lung volumes pattern was detected in 3 out of 35 patients, 2 with LCI >95° pc of controls ~~and one with a borderline high LCI~~ (figure 4). In these subjects, the increased ventilation inhomogeneity was more likely to be due to conductive airway impairment rather than to peripheral lung damage, showing another aspect of the spectrum of manifestations of SCA-related chronic lung disease

Strengths and limitations

The main strength of this study is the use of MBW to assess respiratory impairment in patients with SCA, which has so far received relatively little attention. The integration of results from different tests allowed a comprehensive evaluation of lung function. Among the MBW indices, the calculation of S_{acin} & S_{cond}, in addition to more commonly used LCI, provided insight into the possible localization of pathology along the airway tree. Moreover, this is among ~~is among~~the first studies~~ies~~ presenting S_{acin} & S_{cond} values obtained by Spiroware 3.3.1 and demonstrating that regional ventilation inhomogeneity indices are not significantly affected by the correction of the error in the nitrogen concentration calculation of the Exhalyzer®D. The presence of a control group matched for ethnicity and age (± 1 year) was essential for the correct interpretation of MBW and body plethysmography findings, in the absence of validated references values for people of African ancestry.

The study also has limitations. Most importantly, the absence of simultaneous lung imaging assessment prevents us from relating the MBW abnormalities to specific structural alterations, which can only be speculated upon. Given the absence of published normative values for N_2MBW in children and adolescents of black African origin, we could only establish the ULN for MBW parameters according to the distribution of values in our control population but we cannot exclude that ULN values derived from a large reference population would have been different, affecting the results. Moreover, the cross-sectional

design does not allow us to draw conclusions regarding the clinical and prognostic meaning of LCI alterations in patients with SCA. It should also be taken into account that a minimal clinically important difference for LCI values has not been established so far, therefore we cannot be certain that the difference found in mean LCI between patients and controls (0.42~~74~~, 95% CI 0.~~45~~ 22 to 0.63~~1.02~~), albeit highly statistically significant (*P* 0.0001), corresponds to relevant differences in terms of respiratory pathophysiology. Differences in S_{acin} values between SCA patients and controls were ~~even smaller than differences in LCI, with only a few of patients having S_{acin} >95° pc of controls (5/34, 15% of the total~~influenced by the presence of a patient with very high S_{acin} (0.170 units), without whom statistical significance would be missed (*p* would change from 0.03 to 0.06). However, a trend towards higher S_{acin} values in SCA patients than in controls would be still evident also without this subject ~~(figure 3, more details in OLS))~~. In view of the above, both the finding of greater ventilation inhomogeneity in SCA patients and the mechanisms driving this will need further confirmation in future studies. The absence of prospective longitudinal data and the small sample size also limited the evaluation of risk factors for pathological MBW results. In particular, the study was not powered for multivariable regression analysis. Furthermore, the use of S_{cond} and S_{acin} as proxies for regional ventilation inhomogeneity is based on assumptions that have not been validated in the pediatric population and, therefore, the interpretation of results cannot be certain. Finally, the study is only representative of subjects with SCA followed at tertiary care centres, who, however, represent the great majority of patients living in high-income countries.

Future directions

Future studies should further investigate whether ventilation inhomogeneity is frequently abnormal in patients with SCA and whether the LCI is useful to track early respiratory disease in young people with this condition. To explore this hypothesis, a prospective

longitudinal study of adequate power and involving monitoring of lung function, chest imaging (*i.e.* chest CT, functional MRI of the lung), clinical events and adjustment for confounders (including therapies received) would be required. If an abnormal LCI were proven to correlate with structural lung abnormalities, more rapid decline of lung function or a worse clinical course, the use of MBW could be considered in the routine respiratory of patients with SCA (as detection of early lung disease would offer a window of opportunity for therapeutic interventions) and/or as possible endpoint in clinical trials evaluating disease-modifying drugs in this group.

Conclusion

This study showed that the MBW is more frequently abnormal than spirometry and body plethysmography in young patients with SCA. We found mildly increased ventilation inhomogeneity in people with SCA, which appears to result in most cases from peripheral or parenchymal lung disease, rather than obstructive disease.

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Figure 1. Lung volumes pattern classification according to spirometry and total lung capacity values in individuals with sickle cell anemia. Modified from *Cohen RT. et al*[9].
Abbreviations: FEV_1 = Forced expired volume in the 1st second; FVC = Forced vital capacity; TLC = Total Lung Capacity; SCA = Sickle cell anemia;
Low = <-1.64 z-scores predicted according to the Global Lung Function Initiative reference values for spirometry[33] ("Black" equation) and static lung volumes[34] with 12% ethnic group correction for TLC.[35]

Figure 2. Study population.
Abbreviations: SCA = sickle cell anemia.

Figure 3. Comparison of lung function outcomes in 35 individuals with sickle cell anemia (SCA, black dots) and 31 healthy controls (white dots) aged 8-21 years. The straight bars indicate the mean values with 95% confidence interval in each group. The dashed lines indicate the limits of normal, corresponding to the 5th percentile of the reference population[33,34] for FEV_1, FVC, FEV_1/FVC and TLC (12% ethnic group correction for TLC[35]) and 95th percentile of the controls for LCI.
Abbreviations**:** SCA = Sickle cell anemia; $zFEV_1$ = Z-score forced expired volume in the 1st second; zFVC = Z-score forced vital capacity; zTLC = Z-score total lung capacity; LCI = Lung clearance index; LLN = Lower limit of normal; ULN = Upper limit of normal

Figure 4. LCI values plotted against FEV_1 z-scores (A), FVC z-scores (B), FEV_1/FVC z-scores (C) and TLC z-scores (D) in 35 individuals with sickle cell anemia (black dots) and 31 healthy controls (white dots) aged 8-21 years. The dashed lines indicate the limits of normal, corresponding to the 5th percentile of the reference population for FEV_1, FVC & FEV_1/FVC z-scores[33] and TLC z-scores[34] (12% ethnic group correction for TLC[35]) and to the 95th percentile of the control group for LCI.
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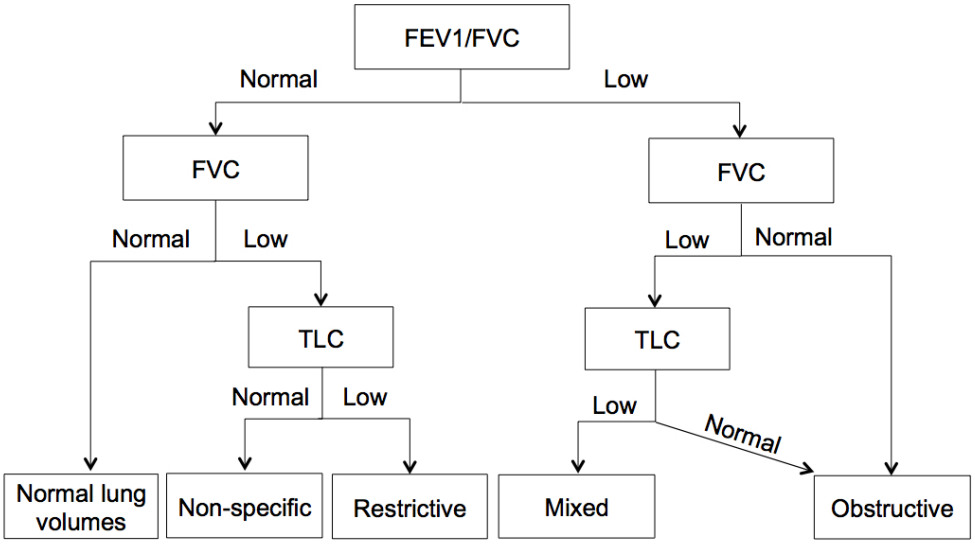

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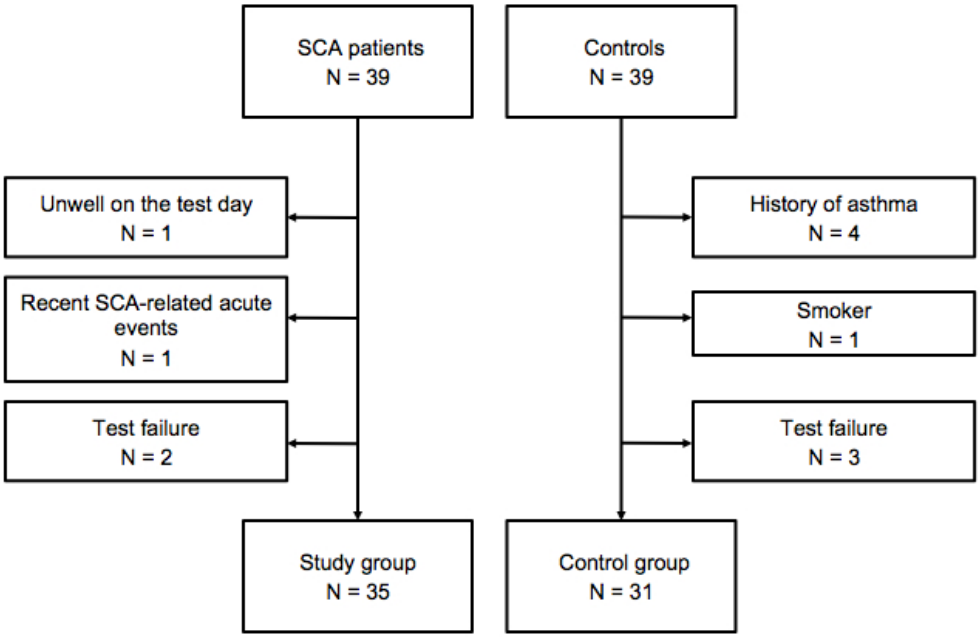

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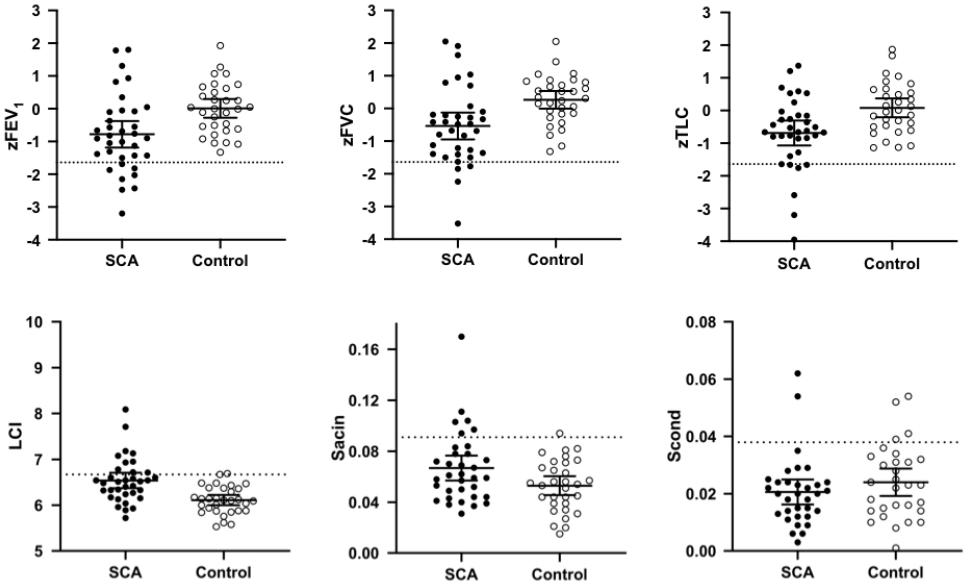

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Lung clearance index might detect early peripheral lung disease in sickle cell anemia

Online supplement

Michele Arigliani, Fenella J. Kirkham, Sati Sahota, Mollie Riley, Ilaria Liguoro, Luigi Castriotta, Atul Gupta, David Rees, Baba Inusa, Paul Aurora.

Methods

Recruitment

Patients who had previously taken part in the prospective observational "Sleep and Asthma Cohort Study" (SAC)[1] and in the pilot phase of a cross-over trial evaluating nocturnal auto-adjusting continuous positive airway pressure and nocturnal oxygen therapy in patients with SCA (POMS2a)[2] were invited to participate. Both studies had enrolled patients with SCA without regard to past morbidity or diagnosis of asthma. A minority of patients were enrolled among relatives of participants in the SAC and POMS2a studies. Controls were recruited among participants of the SAC & POMS2a studies and

among friends and relatives of participants. Controls with a history of asthma (family reported doctor's diagnosis) were excluded as normative values from healthy subjects were needed for Multiple Breath Washout (MBW).

Questionnaire

Caregivers and participants were interviewed via a questionnaire investigating: a) demographics, smoking status and exposure to environmental tobacco smoke; b) presence of current asthma ("wheezing or whistling in the chest in the past 12 months and/or current prescription of anti-asthma medication") and family reported previous doctor's diagnosis of asthma; c) history of acute chest syndrome (ACS) or stroke; d) number of vaso-occlusive pain episodes (VOEs, acute pain lasting at least 4 hours with no explanation other than vaso-occlusion requiring urgent medical evaluation) in the last year; e) current medication.

Assessments

Standing height was measured to the nearest mm using a calibrated stadiometer (Seca 264 stadiometer, Seca, Birmingham, UK). Weight was measured in light clothing and without shoes to the nearest 0.1 kg using a digital scale (Marsden M-11 column scale, Marsden WMG Ltd, Rotherham, UK).

Lung function tests and quality control were performed by an experienced investigator and were over-read by a senior respiratory physiologist to ensure validity of the results.

Prior to each testing day, the N_2MBW device and plethysmograph were calibrated in accordance with the manufacturer's instructions.

For the N_2MBW, temperature and pressure conditions were verified and recorded in the software. The pre-capillary dead space was 29.5 ml (measured using water displacement at study site) for all tests performed with both setup 2 (subjects ≤35kg) and setup 3 (subjects >35kg). The post-capillary dead space was 9.5 ml for set 2 and 22 ml for set 3. Each participant was tested in a single visit and performed at least three N_2MBW trials, with at least two acceptable trials used in analysis. The N_2MBW was performed in the upright seated position, with a nose clip *in situ* and while breathing at tidal volume through a mouth piece connected to a bacterial filter (Air Safety Eco Slimline, No. 4222/01), spirette, and appropriate dead space reducer. Participants were asked to perform regular, quiet breathing. The washout was stopped following at least 3 breaths below 1/40th of the pre-phase end-tidal N_2 concentration.[3] Next MBW trial was started only when end-tidal N_2 levels returned to baseline.

S_{cond} reflects convention dependent ventilation inhomogeneity (CDI), resulting from differences in specific ventilation and in the sequential filling and emptying among lung units sharing branch points within the conductive airways (figure S1). S_{acin} reflects diffusion-convention dependent (DCDI), determined by differences in the emptying rates of the lung units at the diffusion-convection front due to structural asymmetry at branch point (*e.g.* unequal narrowing of parallel airway branches due to disease process, or different volumes/difference in lung compliance of lung regions subtended to different branches).[4] The DCDI, contributes greatly to overall inhomogeneity at the beginning of a MBW trial, but remains stable after the fifth breath (or 1.5 TO).[5]

A graphic representation of the MBW index S_{cond} is provided in figure S1.

Results

The multivariable regression models for LCI and S_{acin} in patients with SCA are presented in table S1.

The only 2 patients with SCA and abnormal S_{cond} values were both known asthmatics (with obstructive spirometry).

Out of 4 SCA patients with current asthma (11%), one had an obstructive pattern, 2 of them had abnormal LCI and S_{cond}. However, at multivariable regression analysis, when

adjusting for other confounders, there was not significant association between current asthma and LCI.

There was no clear trend of FEV_1 and MBW outcomes with increasing age (figure S2).

Table S2 shows differences in N_2MBW outcomes when re-analysing the data with v.3.3.1 Spiroware software version for Exhalyzer®D as compared to the older v.3.1.6 that was originally used to collect the data.

DISCUSSION

We found a moderate inverse correlation between LCI, FEV_1&FVC and TLC, with reduced static & dynamic lung volumes (albeit still within the limit of normal) in the majority of SCA patients with LCI >95° pc (figure 4). The findings suggest that in these subjects increased ventilation inhomogeneity might be an early sign of peripheral lung disease with patchy distribution (otherwise we would not expect a significant impact on LCI).

We could not demonstrate in this study a relationship between increasing age of the SCA patients and worsening spirometry and MBW results (figure S2). This is likely to depend on the cross-sectional nature of the study that does not allow to appreciate changes in lung function trajectory over time in the same patient.

The use of hydroxyurea was not associated with better outcomes at MBW. However, an adequate evaluation of the impact of hydroxyurea on lung function requires

necessarily longitudinal data as this treatment is generally initiated in the sickest patients that might also have worse lung function. Preliminary evidence from longitudinal studies suggests a beneficial effect of hydroxyurea on the rate of FEV_1 decline[8].

There was an outlier value for S_{acin} among patients with SCA (figure 3). Eliminating this subject from the analysis would result in a minimal reduction of mean S_{acin} in the SCA group from 0.067±0.028 to 0.064±0.022 but in a loss of statistical significance for differences in mean S_{acin} values between SCA patients and controls (*P* from 0.03 to 0.06). However, given the very tiny reduction in mean S_{acin} value in SCA group without the outlier (only 0.003 units) and the evident trend of higher S_{acin} values in the SCA group compared with controls in figure 3 (as opposite to what we see for S_{cond}), we think that this finding should not affect the interpretation of results, that is DCDI and peripheral lung disease are more likely to be the cause of increased ventilation inhomogeneity in SCA patients compared with CDI and conductive airways disease.

Among spirometric indices, we did not consider the forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$). The reason is that for measures of flows at a percentage of the FVC, such as $FEF_{25–75\%}$, if FVC and total lung capacity are affected by disease, as may occur in people with SCA (20% of our study group had a TLC <5° pc), forced expiratory

flows will be measured at a different lung volume than in healthy subjects, preventing the possibility of comparison with controls and accurate interpretation of results.[7]

Table S1. Multivariable linear regression models for LCI (35 subjects) and S_{acin} (34 subjects) in the patients with SCA aged 8-21 years.

Covariate	1/LCI[#] Adjusted B[¶] (95% CI; *p*-value)	$1/S_{acin}$[#] Adjusted B[¶] (95% CI; *p*-value)
Male sex	0.0008 (-0.008 to 0.009; 0.84)	4.6 (0.41 to 8.8; 0.03)
Age, yr	0.0003 (-0.000 to 0.0015; 0.61)	0.4 (-0.16 to 0.96; 0.2)
Current asthma[#]	0.003 (-0.009 to 0.017; 0.054)	5.1 (-1.06 to 11.4; 0.1)
Hydroxyurea	-0.004 (-0.004 to 0.004; 0.37)	-0.73 (-6.2 to 3.0; 0.49)
Previous ACS [§]	-0.002 (-0.01 to 0.006; 0.7)	**-5.6 (-9.7 to -1.53; 0.009)**

Abbreviations. ACS: acute chest syndrome

[¶]Adjusted R^2 of the models: -0.01 for 1/LCI and 0.24 for $1/S_{acin}$

[#]The choice to present LCI and S_{acin} as inverted outcomes depended on the fact that the models for LCI and S_{acin} did not satisfy the assumptions of regression.

[#] Wheezing or whistling in the chest in the past 12 months and/or current prescription of anti-asthma medication

[§] Fever and/or respiratory symptoms, plus a new pulmonary infiltrate on a chest x-ray[9]

Table S2. Summary of the differences in lung clearance index, functional residual capacity and regional ventilation inhomogeneity between patients with SCA and controls when using the old Spiroware v.3.1.6 & the new Spiroware v3.3.1 for Exhalyzer®D N_2MBW

		Spiroware 3.1.6	**Spiroware 3.3.1**	**Difference**		
LCI (TO)	*n*			Mean	Rel., %	95% CI %, (*p*)
SCA	35	7.61 (0.71)	6.53 (0.49)	1.08	14.1	1 to 17 (<0.001)
HC	31	6.87 (0.39)	6.11 (0.30)	0.77	11.1	8 to 13 (<0.001)
Difference		0.74 (*p* <0.001)	0.42 (p <0.001)			
FRC, L						
SCA	35	2.07 (0.71)	1.88 (0.66)	0.19	9.2	-6 to 25 (0.25)
HC	31	2.29 (0.83)	2.11 (0.14)	0.18	8.8	-10 to 25 (0.40)
Difference		-0.22 (*p* = *0.3*)	-0.23 (*p* = 0.2)			
S_{acin}						
SCA	34	0.067 (0.027)	0.067 (0.028)	0	0	-1 to 1 (0.9)
HC	31	0.054 (0.020)	0.053 (0.020)	0.001	0	-17 to 20 (0.7)
Difference		0.013 (*p* = 0.04)	0.014 (*p* = 0.03)			
S_{cond}						95% CI %, (*p*)
SCA	33	0.019 (0.009)	0.021 (0.012)	-0.002	-10%	-37 to 21 (0.6)
HC	30	0.021 (0.008)	0.024 (0.013)	-0.003	-14%	-38 to 15 (0.3)
Difference		-0.002 (*p* = 0.4)	-0.003 (*p* = 0.3)			

Definition of abbreviations = SCA, Sickle cell anemia; HC, healthy controls; LCI, lung clearance index; Rel., relative difference; TO, turnover; *n*: the number of subjects; FRC, functional residual capacity; Spiroware 3.1.6 & 3.3.1 are software versions for the Exhalyzer®D for N_2MBW

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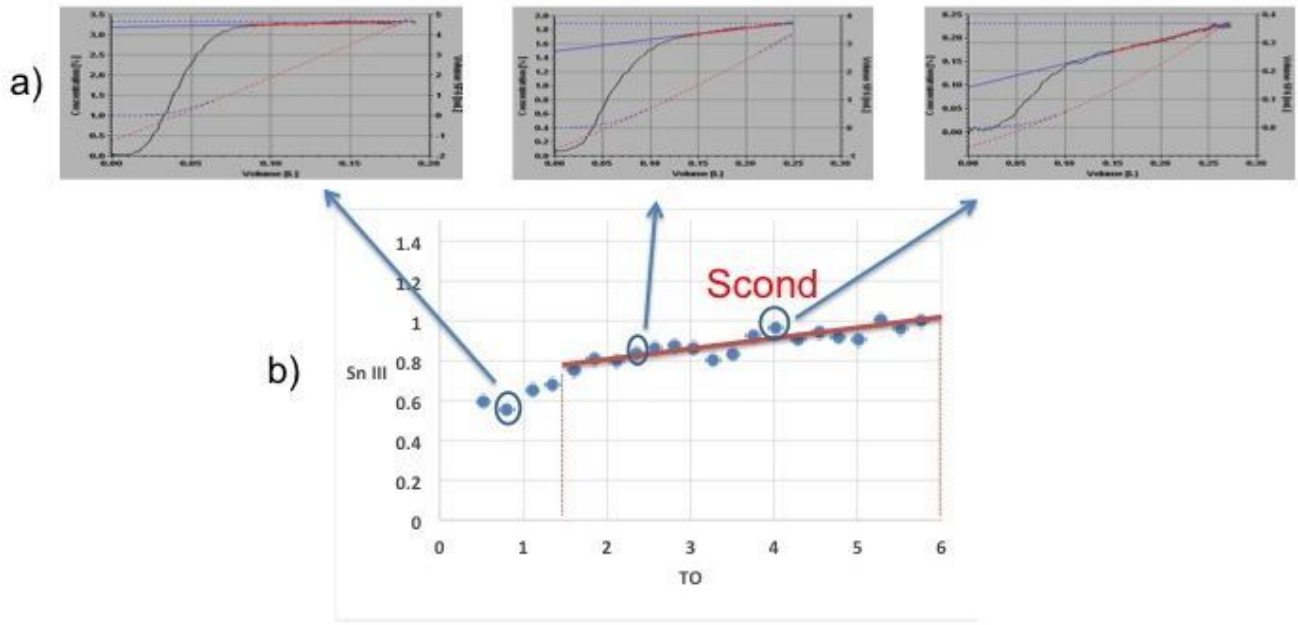

Figure S1. Graphic representation of S_{cond} index from Multiple Breath Washout (MBW). Although this was a SF_6 MBW performed with an Amis 2000 respiratory mass spectrometer, the explication of S_{cond} is fully applicable to the N_2MBW obtained by the Exhalyzer D.

a) Three expirograms of breaths at different point of the MBW are shown. The phase III slope is, numerically, the coefficient of the (red) linear regression of the tracer gas concentration (*y* axis) vs expired volume (*x* axis) in the alveolar phase III (55-95% of the expired volume), for each breath of the MBW. The alveolar slope is divided by mean expired nitrogren (N_2) concentration over the phase III interval and multiplied by the expiratory tidal volume of the breath in litres, in order to give a normalized alveolar slope (S_{nIII}). In ideal conditions, the phase III slope is flat as the N_2 from all the lung units reaches the mouth simultaneously. However, in subjects with ventilation inhomogeinity the

washout of the sickest lung units is delayed and S_{nIII} values progressively increases throughout the washout.

b) S_{nIII} values for each breath are plotted against their corresponding lung volume turnover (TO; 1 TO = cumulative expired volume that equals the FRC). S_{cond} is taught to reflect convenction-dependent inhomogeneity (CDI) arising within the airways proximal to acinar zones, which is predicted to increase linearly through the course of a MBW.[12,13] It is obtained by the calculated S_{nIII} increase between 1.5 and 6.0 TO of the washout.[3] S_{acin}, instead, is intended to reflect diffusion-convenction dependent ventilation inhomogeneity (DCDI) at the entry of the acinus, which is predicted to contribute greatly to ventilation inhomogeneity at beginning of the MBW (approximately 80% of contribution in the first breath), reaching an asymptote by the fifth breath (1.5 TO).[12,13] S_{acin} is computed by the S_{nIII} of the first breath of the washout minus the S_{cond} contribution to its S_{nIII} value.[3] The theoretical background behind these indices that were derived by lung modelling and experimental studies in adults is explicated in detail elsewhere.[4] This figure was originally presented in the online supplement of a previous paper by the first author[14] and it is re-proposed here to facilitate the understanding of S_{cond}.

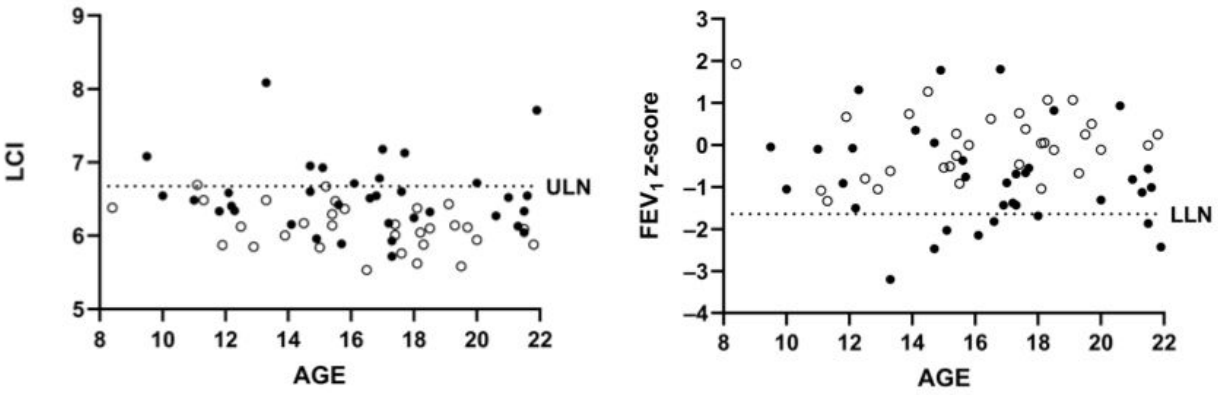

Figure S2. Scatter plots of FEV_1 vs age and LCI vs age in 35 individuals with sickle cell anemia (black dots) and 31 healthy controls (white dots) aged 8-21 years. The dashed lines indicate the limits of normal, corresponding to the 5th percentile of the reference population for FEV_1[10] and to the 95th percentile of the control group for LCI

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Lung clearance index might detect early peripheral lung disease in sickle cell anemia

Online supplement

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The multivariable regression models for LCI and S_{acin} in patients with SCA are presented in table S1.

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Out of 4 SCA patients with current asthma (11%), one had an obstructive pattern, 2 of them had abnormal LCI and S_{cond}. However, at multivariable regression analysis, when adjusting for other confounders, there was not significant association between current asthma and LCI.

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depend on the cross-sectional nature of the study that does not allow to appreciate changes in lung function trajectory over time in the same patient.

The use of hydroxyurea was not associated with better outcomes at MBW. However, an adequate evaluation of the impact of hydroxyurea on lung function requires necessarily longitudinal data as this treatment is generally initiated in the sickest patients that might also have worse lung function. Preliminary evidence from longitudinal studies suggests a beneficial effect of hydroxyurea on the rate of FEV_1 decline[8].

There was an outlier value for S_{acin} among patients with SCA (figure 3). Eliminating this subject from the analysis would result in a minimal reduction of mean S_{acin} in the SCA group from 0.067±0.028~~7~~ to 0.064±0.0~~20~~ 022 but in a loss of statistical significance for differences in mean S_{acin} values between SCA patients and controls (*P* from 0.03~~4~~ to 0.06~~7~~). However, given the very tiny reduction in mean S_{acin} value in SCA group without the outlier (only 0.003 units) and the evident trend of higher S_{acin} values in the SCA group compared with controls in figure 3 (as opposite to what we see for S_{cond}), we think that this finding should not affect the interpretation of results, that is DCDI and peripheral lung disease are more likely to be the cause of increased ventilation inhomogeneity in SCA patients compared with CDI and conductive airways disease.

Among spirometric indices, we did not consider the forced expiratory flow at 25–75% of FVC ($FEF_{25–75\%}$). The reason is that for measures of flows at a percentage of the FVC, such as $FEF_{25–75\%}$, if FVC and total lung capacity are affected by disease, as may occur in people with SCA (20% of our study group had a TLC <5° pc), forced expiratory flows will be measured at a different lung volume than in healthy subjects, preventing the possibility of comparison with controls and accurate interpretation of results.[7]

Table S1. Multivariable linear regression models for LCI (35 subjects) and S_{acin} (34 subjects) in the patients with SCA aged 8-21 years.

Covariate	1/LCI[#] Adjusted B[¶] (95% CI; *p*-value)	1/S_{acin}[#] Adjusted B[¶] (95% CI; *p*-value)
Male sex	0.00081 (-0.0086 to 0.0091; 0.8497)	43.67 (0.41-0.65 to 8.80; 0.039)
Age, yr	0.00035 (-0.00006 to 0.001502; 0.6136)	0.4 (-0.165 to 0.961.0; 0.213)
Current asthma[#]	0.0031 (-0.0092 to 0.01721; 0.0545)	1.355.1 (-1.064.0 to 11.46.7; 0.163)
Hydroxyurea	-0.0048 (-0.00417 to 0.00405; 0.3706)	-0.73 (-6.2-5.6 to 3.04.2; 0.4976)
Previous ACS[§]	-0.00209 (-0.0108 to 0.0068; 0.798)	**-5.65 (-9.78 to -1.53.28; 0.00913)**

Abbreviations. ACS: acute chest syndrome

¶Adjusted R^2 of the models: -0.010.09 for 1/LCI~~zFEV$_1$~~ and 0.2415 for 1/S_{acin}

#The choice to present LCI and S_{acin} as inverted outcomes depended on the fact that the models for LCI and S_{acin} did not satisfy the assumptions of regression.

Wheezing or whistling in the chest in the past 12 months and/or current

prescription of anti-asthma medication

§ Fever and/or respiratory symptoms, plus a new pulmonary infiltrate on a chest x-ray[9]

Table S2. Summary of the differences in lung clearance index, functional residual capacity and regional ventilation inhomogeneity between patients with SCA and controls when using the old Spiroware v.3.1.6 & the new Spiroware v3.3.1 for Exhalyzer®D N_2MBW

		Spiroware 3.1.6	**Spiroware 3.3.1**	**Difference**		
LCI (TO)	*n*			Mean	Rel., %	95% CI %, (*p*)
SCA	35	7.61 (0.71)	6.53 (0.49)	1.08	14.1	1 to 17 (<0.001)
HC	31	6.87 (0.39)	6.11 (0.30)	0.77	11.1	8 to 13 (<0.001)
Difference		0.74 (*p* <0.001)	0.42 (p <0.001)			
FRC, L						
SCA	35	2.07 (0.71)	1.88 (0.66)	0.19	9.2	-6 to 25 (0.25)
HC	31	2.29 (0.83)	2.11 (0.14)	0.18	8.8	-10 to 25 (0.40)
Difference		-0.22 (*p = 0.3*)	-0.23 (*p* = 0.2)			
S_{acin}						
SCA	34	0.067 (0.027)	0.067 (0.028)	0	0	-1 to 1 (0.9)
HC	31	0.054 (0.020)	0.053 (0.020)	0.001	0	-17 to 20 (0.7)
Difference		0.013 (*p* = 0.04)	0.014 (*p* = 0.03)			
S_{cond}						95% CI %, (*p*)
SCA	33	0.019 (0.009)	0.021 (0.012)	-0.002	-10%	-37 to 21 (0.6)
HC	30	0.021 (0.008)	0.024 (0.013)	-0.003	-14%	-38 to 15 (0.3)
Difference		-0.002 (*p* = 0.4)	-0.003 (*p* = 0.3)			

Definition of abbreviations = SCA, Sickle cell anemia; HC, healthy controls; LCI, lung clearance index; Rel., relative difference; TO, turnover; *n*: the number of subjects; FRC, functional residual capacity; Spiroware 3.1.6 & 3.3.1 are software versions for the Exhalyzer®D for N_2MBW

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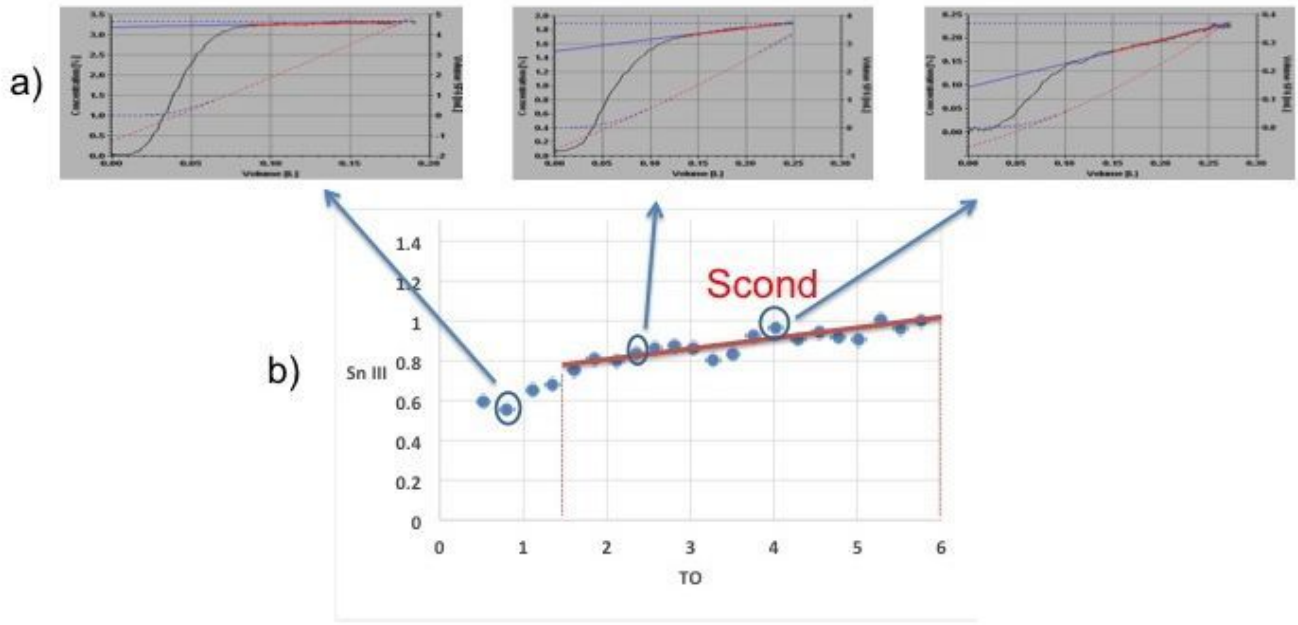

Figure S1. Graphic representation of S_{cond} index from Multiple Breath Washout (MBW). Although this was a SF_6 MBW performed with an Amis 2000 respiratory mass spectrometer, the explication of S_{cond} is fully applicable to the N_2MBW obtained by the Exhalyzer D.

a) Three expirograms of breaths at different point of the MBW are shown. The phase III slope is, numerically, the coefficient of the (red) linear regression of the tracer gas concentration (*y* axis) vs expired volume (*x* axis) in the alveolar phase III (55-95% of the expired volume), for each breath of the MBW. The alveolar slope is divided by mean expired nitrogren (N_2) concentration over the phase III interval and multiplied by the expiratory tidal volume of the breath in litres, in order to give a normalized alveolar slope (S_{nIII}). In ideal conditions, the phase III slope is flat as the N_2 from all the lung units reaches the mouth simultaneously. However, in subjects with ventilation inhomogeinity the

washout of the sickest lung units is delayed and S_{nIII} values progressively increases throughout the washout.

b) S_{nIII} values for each breath are plotted against their corresponding lung volume turnover (TO; 1 TO = cumulative expired volume that equals the FRC). S_{cond} is taught to reflect convenction-dependent inhomogeneity (CDI) arising within the airways proximal to acinar zones, which is predicted to increase linearly through the course of a MBW.[12,13] It is obtained by the calculated S_{nIII} increase between 1.5 and 6.0 TO of the washout.[3] S_{acin}, instead, is intended to reflect diffusion-convenction dependent ventilation inhomogeneity (DCDI) at the entry of the acinus, which is predicted to contribute greatly to ventilation inhomogeneity at beginning of the MBW (approximately 80% of contribution in the first breath), reaching an asymptote by the fifth breath (1.5 TO).[12,13] S_{acin} is computed by the S_{nIII} of the first breath of the washout minus the S_{cond} contribution to its S_{nIII} value.[3] The theoretical background behind these indices that were derived by lung modelling and experimental studies in adults is explicated in detail elsewhere.[4] This figure was originally presented in the online supplement of a previous paper by the first author[14] and it is re-proposed here to facilitate the understanding of S_{cond}.

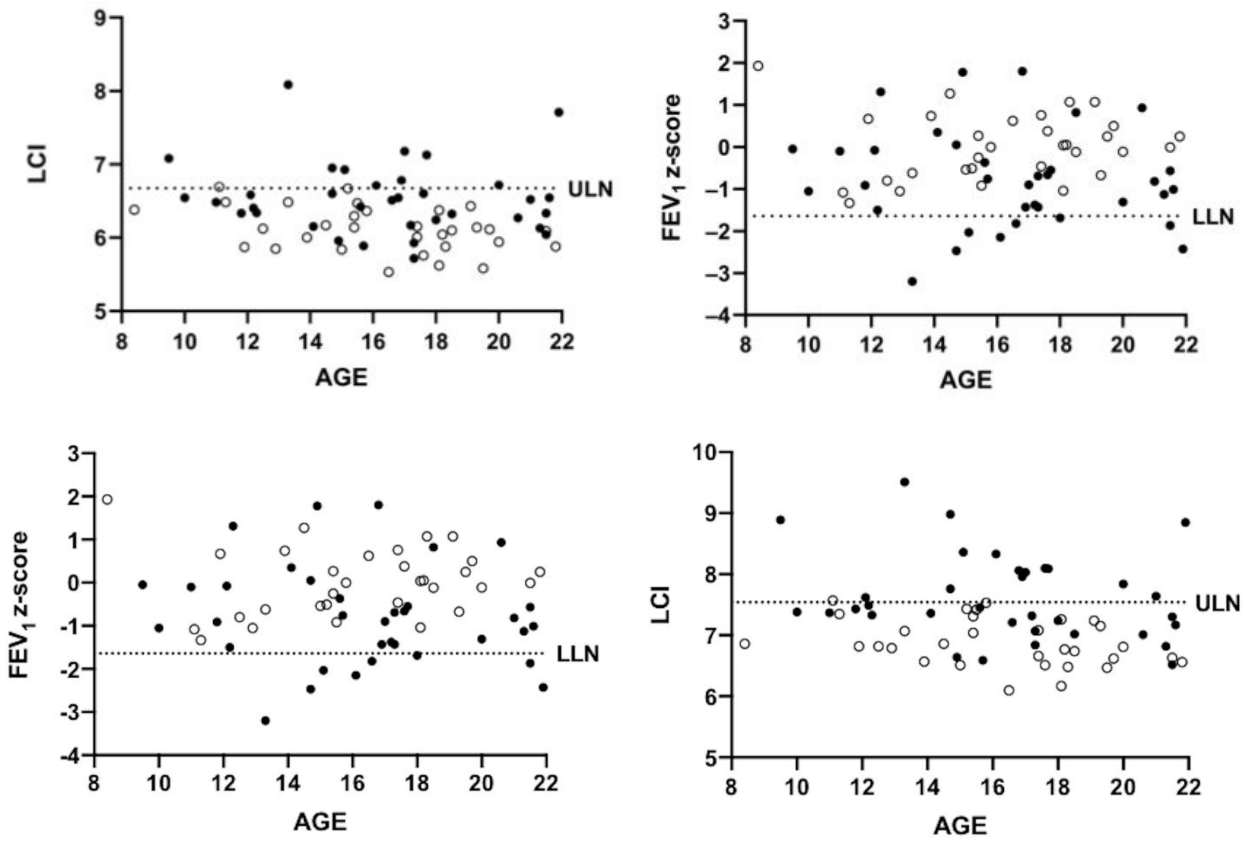

Figure S2. Scatter plots of FEV_1 vs age and LCI vs age in 35 individuals with sickle cell anemia (black dots) and 31 healthy controls (white dots) aged 8-21 years. The dashed lines indicate the limits of normal, corresponding to the 5th percentile of the reference population for FEV_1[10] and to the 95th percentile of the control group for LCI
Abbreviations: LCI = Lung clearance index; FEV_1 = forced expired volume in the 1st second LLN = Lower limit of normal; ULN = Upper limit of normal