Pediatric RESEARCH

SYSTEMATIC REVIEW

Exhaled volatile organic compounds analysis in clinical pediatrics: a systematic review

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BACKGROUND: Measured exhaled volatile organic compounds (VOCs) in breath also referred to as exhaled volatilome have been long claimed as a potential source of non-invasive and clinically applicable biomarkers. However, the feasibility of using exhaled volatilome in clinical practice remains to be demonstrated, particularly in pediatrics where the need for improved non-invasive diagnostic and monitoring methods is most urgent. This work presents the first formal evidence-based judgment of the clinical potential of breath volatilome in the pediatric population.
METHODS: A rigorous systematic review across Web of Science, SCOPUS, and PubMed databases following the PRISMA statement guidelines. A narrative synthesis of the evidence was conducted and QUADAS-2 was used to assess the quality of selected studies.
RESULTS: Two independent reviewers deemed 22 out of the 229 records initially found to satisfy inclusion criteria. A summary of breath VOCs found to be relevant for several respiratory, infectious, and metabolic pathologies was conducted. In addition, we assessed their associated metabolism coverage through a functional characterization analysis.
CONCLUSION: Our results indicate that current research remains stagnant in a preclinical exploratory setting. Designing exploratory experiments in compliance with metabolomics practice should drive forward the clinical translation of VOCs breath analysis.

Pediatric Research (2021) 89:1352–1363; https://doi.org/10.1038/s41390-020-01116-8

IMPACT:

- What is the key message of your article?
  Metabolomics practice could help to achieve the clinical utility of exhaled volatilome analysis.
- What does it add to the existing literature?
  This work is the first systematic review focused on disease status discrimination using analysis of exhaled breath in the pediatric population. A summary of the reported exhaled volatile organic compounds is conducted together with a functional characterization analysis.
- What is the impact?
  Having noted challenges preventing the clinical translation, we summary metabolomics practices and the experimental designs that are closer to clinical practice to create a framework to guide future trials.

INTRODUCTION

A wide range of volatile organic compounds (VOCs) is present in different specimens of the human body such as in skin emanations, urine, blood, saliva, feces, and exhaled breath.[1] The blend of VOCs present in exhaled breath can provide insights into the metabolic status of an individual and therefore breath volatilome analysis holds promise for clinical diagnosis and therapeutic monitoring.[2–4] Breath VOCs analysis has been used in the adult population to search for disease markers in pathologies such as asthma,[5] chronic obstructive pulmonary disease (COPD),[6] cystic fibrosis (CF),[7,8] lung cancer,[9,10] colorectal cancer,[11] gastric carcinoma,[12] thyroid cancer,[13] tuberculosis,[14] liver cirrhosis,[15] and type 2 diabetes mellitus,[16] among others. In addition, they have also been used to discriminate disease stages or exacerbations.[17–19] Noteworthy, breath volatilome can be directly obtained at the point of care in a rather simple and straightforward manner with unlimited sample availability and using non-invasive sampling methods.[4] This places breath VOCs as an ideal biospecimen to work within the pediatrics population where they have lately received substantial interest for early diagnosis and surveillance of childhood pathologies most of them becoming chronic diseases imposing large socio-economic burden.[20–22] For example, VOCs breath analysis can potentially add to asthma diagnosis, which remains yet challenging due to the inherent low sensitivity associated with spirometry with bronchodilation.[23] Thus, breath volatilome is expected to become

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Received: 21 February 2020 Revised: 9 July 2020 Accepted: 4 August 2020
Published online: 12 September 2020

more widely adopted for diagnosis in very young non-collaborative children.[24] However, despite all these initial promising results, breath volatilome analysis has had to date little or no progress from the laboratory to the clinical setting. This has largely been attributed to the lack of standardization in breath collection, profiling detection platforms, and robust data analysis.[20,25] Notwithstanding, the breath research community is enthusiastically striving to place breath analysis as a routine clinical tool by implementing new technologies and developing a community consensus for standardization.[26] On the other side, the medical community is asking whether current technically demanding analytical methods to measure breath VOCs pay off in terms of feasibility and reproducibility and question the translation to clinical practice.[27] VOCs breath analysis in pediatrics remains poorly studied and conclusions about its clinical feasibility remain elusive and controversial. In the case of childhood asthma for example, whereas VOCs measurements have been found of limited clinical value in some studies, they have shown moderate to good prediction accuracy for pediatric asthma diagnosis and some potential to predict asthma exacerbations.[28–30] The clinical potential of breath VOCs test is thus under debate. With the aim to clarify whether the currently available body of evidence is reliable enough to support claiming breath VOCs clinical potential in pediatrics, here we present the first systematic review on their use for clinical diagnosis or therapeutic monitoring of childhood diseases.

METHODS

Protocol and eligibility criteria

This systematic review was performed according to the PRISMA statement guidelines.[31] The protocol was registered on PROSPERO (CRD42020151186). The primary review question was aimed to clarify whether it is currently feasible using breath VOCs for the diagnosis and/or therapeutic monitoring of diseases in children and adolescents. Only original articles published in English prior to September 2019 were considered. Studies were deemed eligible if they measured exhaled VOCs to either discriminate disease status or monitor pathologies and the median age of the study population was less than 18 years. Duplicate or redundant publications were excluded.

Search strategy

Web of Science, SCOPUS, and PubMed databases were considered. PubMed search strategy consisted of a combination of the following keywords: ((("exhaled") AND ("VOC" OR "VOCs" OR "volatile organic compound" OR "volatile organic compounds" OR "breathomics" OR "volatilome" OR "exhaled metabolites")) AND ("children" OR "childhood" OR "baby" OR "babies" OR "teenager" OR "teenagers" OR "adolescent" OR "adolescents" OR "child" OR "infant")) AND ("disease" OR "diseases" OR "pathology" OR "pathologies" OR "patient" OR "patients" OR "pathophysiology" OR "diagnosis" OR "diagnostic" OR "biomarker" OR "biomarkers" OR "marker" OR "markers" OR "monitoring" OR "monitorization"). Minor adjustments of this search strategy were used for Web of Science and SCOPUS databases (Supplementary Table S1).

Study selection

The selection of studies was according to the PRISMA flow-chart,[31] and it was performed independently by two reviewers (RASM and JMPH). Disagreements were resolved by consensus.

Data extraction

The following data were systematically extracted from each study: (i) study design (disease monitoring, disease status); (ii) study population (pathology, sample size, and age); (iii) breath collection (breath collector type, exhaled breath portion, and strategies used to minimize the influence of environmental VOCs); (iv) analytical platform type; (v) statistical analysis; (vi) results (in terms of the number of selected VOCs, and results of classification rate, sensitivity, and specificity); and (vii) identified VOCs reported to be relevant for diagnosis and/or disease monitoring.

Synthesis

Meta-analysis was deemed unfeasible due to the considerable heterogeneity among study populations and VOCs profiles. A descriptive and narrative synthesis of the evidence was conducted instead. This included a comprehensive description of reviewed VOCs and their functional enrichment analysis based on "FELLA" R package.[32,33]

Quality assessment

Two independent reviewers (RASM and JMPH) assessed the quality of the included studies using the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) tool. QUADAS-2 includes four domains (patient selection, index test, reference standard, and flow and timing), and is performed in four phases: (1) report the review question, (2) tailor questions to the features of the review, (3) design a flow diagram, and (4) judge on bias and applicability. For the risk of bias assessment, the reviewers carefully read all selected articles and answered each signaling question with yes, no, or unclear. Supplementary Table S2 summarizes the signaling questions used in this review. Those referred to Domain 2 (Index Test) were modified during Phase 2 (Review-Specific Tailoring). In addition, just the two first signaling questions of Domain 1 (Patient Selection) were taken into account, as they were considered the best suited to answer the main review question. The rest of the domains were left as described in the original QUADAS-2. The concern regarding the applicability of the different domains and were graded low, high, or unclear.[34] Cohen's kappa (*k*) computed using "psych" package[33] was used to test the level of agreement in the responses of both reviewers. Disagreements were resolved by consensus.

RESULTS

Study selection

Overall 229 records were found according to our search strategy. After removing duplicate entries, 125 articles were further screened. Among these, only 43 records presented an abstract that fulfilled the review's criteria and were fully read. An article aimed at improving asthma diagnosis combining exhaled VOCs, gene expression, and lung function measurements was excluded because VOCs measurements were reused from a previous study.[35] Figure 1 depicts the flow diagram of the PRISMA-oriented record search and selection. Accordingly, only 22 articles fulfilled our inclusion/exclusion criteria and were included in the qualitative synthesis:[30,36–56]

Study characteristics

Data from the 22 studies included in the qualitative synthesis was reviewed thoroughly and extracted information is summarized in Table 1. The study population comprised groups of children and adolescents with an average or median age of <18 years. These studies dealt with respiratory diseases being asthma the most represented one (11 out of the 22 records) and to a lesser extent other respiratory diseases such as cystic fibrosis (CF), obstructive sleep apnea syndrome (OSAS), or primary ciliary dyskinesia (PCD). However, this systematic review was not only limited to respiratory diseases but other disorders where VOCs breath analysis was used as either diagnosis or surveillance tool were considered too. These include both infectious (malaria, rhinovirus infection, and bacterial infection in patients with respiratory diseases) and metabolic diseases such as nonalcoholic fatty liver disease (NALFD), chronic liver disease (CLD), chronic kidney disease (CKD), and inflammatory bowel disease (IBD). Among

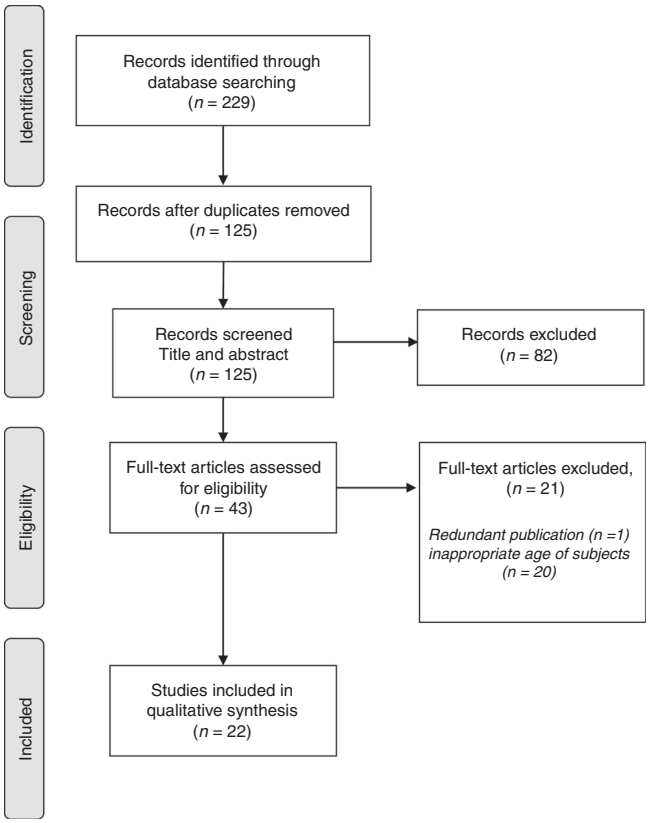

Fig. 1 Selection and inclusion of studies. Flow diagram of PRISMA-oriented systematic search and literature records selection.

the three main disease groups (respiratory, infectious, and metabolic diseases), analysis of VOCs was used to distinguish patients with a certain disease and controls, to predict different disease status or exacerbations, and to discriminate between diseases with similar symptoms and different etiologies. The majority of the studies included a cross-sectional design (73%) and the rest of them were designed as longitudinal studies.

Breath sampling portion and breath collectors

Both breath sampling portion and breath collectors differ across all studies included in our systematic review (Table 1). The portion of the breath is usually classified in late expiratory, end-tidal, and mixed expiratory.[57] Mixed expiratory breath consists of complete exhaled breath collection including death space air whereas late expiratory and end-tidal breath comprises the last breath portion. More than half of the studies included in this systematic review used mixed expiratory breath as a targeted exhaled breath portion. This is probably because the simplicity of breath sampling is the priority for non-collaborative passive patients such as young children. Following mixed expiratory, late expiratory breath portion was used in 6 out of the 22 included studies whereas end-tidal breath was used in just 2 of them. The main difference between late expiratory and end-tidal breath is the way in which the targeted fraction is selected. In late expiratory breathing, the first portion of the breath is discarded. In end-tidal breathing, CO_2[53] or acetone[37] levels are used as indicators of the collected portion. Mixed expiratory breath is simpler to collect than other breath types though it is heavily influenced by exogenous environmental contaminants.[57] Hence, the collection of mixed expiratory breath is usually accompanied by different strategies to reduce the influence of VOCs derived from exogenous sources. Room air sampling is a well-suited control to thoroughly check for exogenous and background compounds in mixed expiratory breath analysis. To quality control breath collection, background compounds and endogenous compounds can be monitored in room air samples and their levels can be compared to those obtained in-breath specimens. In this regard, Schaber and co-authors[54] monitored acetone and isoprene levels (two of the most common and abundant endogenous breath VOCs). Mixed expiratory breath collection combined with room air sampling is a suitable sample collection in non-collaborative infants younger than 2 years provided that background contamination is monitored. Concerning collectors, polymer (Tedlar®) bags followed by aluminized (Mylar®) bags were the most widely used (Table 1). Flushing an inert gas such as nitrogen through Tedlar® bags before sampling was demonstrated as an effective tool to reduce levels of phenol and *N*,*N*-dimethylacetamide (two intrinsic contaminants in Tedlar® bags) and avoid cross-contamination in reused bags.[45] Other collectors such as BIOVOC®[45] were also used to a lesser extent. Beyond breath VOCs, the fraction with lower volatility contained in exhaled breath condensate (EBC) has also proven to be a suitable matrix to help in discriminating persistent asthma in children and adolescents, overcoming the sensitivity of traditional spirometry with bronchodilation test.[23]

Breath VOCs analytical platforms

Breath VOCs analysis in the reviewed studies was performed using different technologies. Mass spectrometry (MS) was used in 17 out of the 22 studies followed far behind by sensor arrays and laser-based spectroscopy used in just 4 and 1 studies, respectively (Table 1). Broadband laser-based cascade quantum spectroscopy remains as an unconsolidated technology with poor repeatability.[41] On the other side, the reduced cost, portability, and ease-to-operate capacities of gas sensor technologies make them a suitable technology for clinical translation of breath analysis.[23,58] However, identification of VOCs is intrinsically not possible and their use is relegated to obtain a breathprint or a pattern of sensor signal responses from a specific VOCs mixture.[59] MS-based analytical platforms offer the highest sensitivity and selectivity and they enable identification and quantification of VOCs in breath usually at the trace levels.[60,61] Both sensitivity and selectivity depend on the particular configuration of each individual mass spectrometry platform. The most common configuration (12 out of the 22 studies included in the qualitative synthesis) consisted of using off-line pre-concentration sampling methods together with GC/MS (gas chromatography coupled to either high- or low-resolution MS). Off-line pre-concentration methods usually involve a two-sequential step procedure. First, breath VOCs are trapped into an active adsorbent (i.e, a solid-phase microextraction (SPME) fibers or packed tubes (e.g., Tenax)) and subsequently these are thermally desorbed to release trapped VOCs to the injection port of a GC-MS system. On the other hand, five studies used technology with inherent on-line breath analysis capacities such as SIFT-MS (selective ion flow tube mass spectrometry), IMR-MS (ion-molecule reaction mass spectrometry), or PTR-MS (proton transfer reaction mass spectrometry). However, just the study using PTR-MS conducted a real-time on-line analysis[37] (Table 1). The remaining ones were considered off-line because all of them implied a breath collection and storage step previous to breath VOCs analysis (Table 1). There are a series of inherent advantages when using off-line pre-concentration methods such as the possibility to perform remote sampling or to tailor different adsorbents to specific VOCs families and the capacity to pre-concentrate VOCs among others.[3,57,61] In addition, this equipment comes to a more affordable cost than the equipment used in on-line breath analysis. However, the real-time on-line analysis does not require any pre-concentration step, which simplifies operational procedures, and therefore it is more amenable for clinical day-to-day diagnosis.[60,62] On-line real-time VOCs breath analysis is nowadays a reality using emerging technologies such as SESI-HRMS (secondary electrospray

Table 1. Information obtained from studies included in the qualitative synthesis.

References	Targeted diseases and study population			Breath sampling		Breath analysis	Data analysis	Results of study
	Disease	Sample size/study design	Age (years)	Ambiental air sample/VOCs filter/sampling in the same room	Exhaled breath portion targeted/ breath container	Analytical methods	Statistical methods	No. of VOCs selected/ classification rate/ sensitivity/specificity
Technologies bases on portable sensors								
[36]	Asthma[a]	A) 20 asthmatics vs. 22 HCTR	Asthmatics: 12.7 ± 3.1 CF: 14.4 ± 4.2 HCTR: 9.7 ± 2.9	Not/yes/yes	Mixed expiratory breath/_	Sensor arrays-three micro hotplate metal-oxide sensors (MOS): Aenose	ANNs	_/AUC = 0.79/74%/ 91%
	CF[a]	B) 13 CF vs. 22 HCTR						_/AUC = 0.87/85%/ 77%
	Asthma vs. CF[a]	C) 20 asthmatics vs. 13 CF						_/AUC = 0.90/89%/ 77%
[39]	CF[a]	A) 25 CF (9 with pulmonary exacerbation) vs. 23 HCTR	CF: 11.4 [7.7, 17.9] PCD: 10.7 [7.1, 14.5] HCTR: 9.3 [5.4, 12.6]	Not/yes/not	Mixed expiratory breath/Spacer Babyhaler	Sensor arrays-array of 32 polymer nanocomposite nanosensors: Cyranose 320	PCR + CDA	_/AUC = 0.76/84%/ 65%
	PCD[a]	B) 25 PCD (4 with pulmonary exacerbation) vs. 23 HCTR						_/AUC = 0.80/88%/ 52%
	CF pulmonary exacerbations[a]	C) 9 CF with pulmonary exacerbation vs. 16 stable CF						_/AUC = 0.76/89%/ 56%
	PDC pulmonary exacerbations[a]	D) 4 PCD with pulmonary exacerbation vs. 19 stable PCD						_/AUC = 0.9/100%/ 90%
	CF vs. PCD[a]	E) 25 CF (9 with pulmonary exacerbation) vs. 25 PCD (4 with pulmonary exacerbation)						_/AUC = 0.77/84%/ 60%
	CF stable disease vs. PCD stable disease[a]	F) 16 stable CF vs. 19 stable PCD						_/AUC = 0.77/95%/ 63%
	S. aureus in CF[b]	G) 13 CF infected with *S. aureus* vs. 12 CF without *S. aureus* infection						X/_/_/_
	H. influenzae in PCD[b]	H) 6 PCD infected with *H. influenzae* vs. 19 PCD without *H. influenzae* infection						X/_/_/_
[42]	Rhinovirus-induced wheeze[b]	A) 37 wheezing symptoms (rhinovirus positive) vs. 26 asymptomatic (rhinovirus positive)	1.8 ± 0.8	Yes/yes/not	Mixed expiratory breath/Spacer Babyhaler	Sensor arrays-array of 32 polymer nanocomposite nanosensors: Cyranose 320	PCA, Student's *t*-test & CDA	_/AUC = 0.77/73%/ 96%
		B) 37 ex-symptomatic (rhinovirus positive) vs. 26 asymptomatic (rhinovirus positive)						_/AUC = 0.84/84%/ 75.9%
	Non-rhinovirus-induced wheeze[a]	C) 44 wheezing symptoms (rhinovirus negative) vs. 71 asymptomatic (rhinovirus negative)						_/AUC = 0.81/75.6%/ 71.4%
		D) 44 ex-symptomatic (rhinovirus negative) vs. 71 asymptomatic (rhinovirus negative)						_/AUC = 0.67/62.9%/ 65.6%
[43]	OSAS[a]	A) 18 OSAS vs.10 snoring CTR	OSAS: 8.2 ± 1.9 Snoring CTR: 9.1 ± 1.6	Yes/yes/yes	Late expiratory breath/(Teflon-coated) Mylar® bag	Sensor arrays-array of 32 polymer nanocomposite nanosensors: Cyranose 320	PCA + LR	4 PCs (PC 3 best discriminant)/AUC = 0.84/78%/70%
Technologies bases on mass spectrometry								
[44]	Asthma[a]	A) 63 asthmatics vs. 57 HCTR	5–16	Not/not/yes	Mixed expiratory breath/Tedlar® bag (5L)	Mass spectrometry -Off-line breath analysis: TD (Carbograph 1TD/Carbopack X)- GC/MS (TOF)	Stepwise discriminant analysis	8 VOCs/92%/85%/95%
	Atopic asthma[a]	B) 42 atopic asthma vs. 57 HCTR						7 VOCs[d]/_/90%/89%
		C) 42 atopic asthma vs. 21 non-atopic asthma						7 VOCs[d]/_/84%/95%
[45]	Allergic asthma[a]	A) 35 allergic asthma (13 with allergic rhinitis) vs. 15 HCTR	4–13	Yes/not/yes	Mixed expiratory breath/Tedlar® bag (1L)	Mass spectrometry-Off-line breath analysis: HS-SPME (DVB/CAR/PDMS)-GC/MS (single quad)	PLS-DA	28 VOCs/88%/_/_

Table 1. continued

References	Targeted diseases and study population			Breath sampling		Breath analysis	Data analysis	Results of study
	Disease	Sample size/study design	Age (years)	Ambiental air sample/VOCs filter/sampling in the same room	Exhaled breath portion targeted/ breath container	Analytical methods	Statistical methods	No. of VOCs selected/ classification rate/ sensitivity/specificity
[46]	Allergic asthma[a]	A) 32 asthmatics (10 with allergic rhinitis) vs. 27 HCTR	Asthmatics: 4–16 HCTR: 3–6	Yes/not/not	Mixed expiratory breath/Tedlar® bag (1L)	Mass spectrometry-Off-line breath analysis: HS-SPME (DVB/CAR/PDMS)-GCxGC/ MS (TOF)	PLS-DA	9 VOCs/96%/98%/93%
[48]	RW[a]	A) 202 recurrent wheezing symptoms vs. 50 no wheezing symptoms	2–4	Not/not/yes	Mixed expiratory breath/Tedlar® bag (1L)	Mass spectrometry-Off-line breath analysis: TD (Carbograph 1TD/Carbopack X)- GC/MS (TOF)	ASCA + SLR	28 VOCs/73%/79%/ 50%
[49]	Asthma[a]	A) 76 asthmatics vs. 49 HCTR	2–4 until 6	Not/not/yes	Mixed expiratory breath/Tedlar® bag (1L)	Mass spectrometry-Off-line breath analysis: TD (Carbograph 1TD/Carbopack X)- GC/MS (TOF)	RF	12 VOCs[d]/85.84%/ 81.5%/74.2%
		B) 76 asthmatics vs. 121 transient wheezers						12 VOCs[d]/77.8%/ 74.6%/70%
			2–4				d-PLS-DA	17 VOCs/80%/_/_
[47]	Asthma[a]	A) 11 asthmatics vs. 12 HCTR	8–16	Yes/not/not	Late expiratory breath/_	Mass spectrometry-Off-line breath analysis: TD (Tenax®/ Carbotrap)-GC/MS (ion-trap)	PLS-DA	8 VOCs/51%/_/_
[50]	Asthmatic exacerbations (intersubject)[a]	A) 40 asthmatics (29 with atopic asthma) - 16 out of 40 asthmatics experienced exacerbations	6–16	Not/not/yes	Mixed expiratory breath/Tedlar® bag (5L)	Mass spectrometry-Off-line breath analysis: TD (Carbograph 1TD/Carbopack X)-GC/MS (TOF)	SVM	7 VOCs/91%/79%/ 100%
	Asthmatic exacerbations (intrasubject)[a]	B) 16 asthmatic exacerbations						6 VOCs/96%/100%/ 93%
[51]	Asthmatic exacerbations[a]	A) 9 persistently uncontrolled vs. 34 persistently controlled asthma	6–18	Not/not/yes	Mixed expiratory breath/Tedlar® bag (5L)	Mass spectrometry-Off-line breath analysis: TD (Carbograph 1TD/Carbopack X)-GC/MS (TOF)	RF	15 VOCs/AUC = 0.86/ 81%/67%
[30]	Asthmatic exacerbations[a]	A) 16 stable vs. 16 asthmatics with an exacerbation 14 days after sampling	6–18	Not/not/yes	Mixed expiratory breath/Tedlar® bag (5L)	Mass spectrometry-Off-line breath analysis: TD (Carbograph 1TD/Carbopack X)-GC/MS (TOF)	RF	7 VOCs/AUC = 0.90/ 88%/75%
[52]	CF[a]	A) 48 CF vs. 57 HCTR	CF: 13.0 ± 0.6 HCTR: 9.9 ± 0.4	Not/not/yes	Mixed expiratory breath/Tedlar® bag (5L)	Mass spectrometry -Off-line breath analysis: TD (Carbograph 1TD/Carbopack X)-GC/MS (TOF)	Stepwise discriminant analysis	10 VOCs/AUC = 0.962/ _/_
	P. aerouginosa in CF[b]	B) 23 CF infected with *P. aerouginosa* vs. 17 CF without *P. aerouginosa* infection						14 VOCs[d]/100%/_/_
[53]	*S. aureus* in CF[b]	A) 13 CF infected with *S. aureus* vs. 5 CF without *S. aureus* infection	*S.aureus* infected CF: 13 [9, 20] CF without *S. aureus* infection: 7 [7, 13]	Yes/yes/not	End-tidal breath/ Tedlar® bag (3L)	Mass spectrometry-Off-line breath analysis: TD (Tenax® TA)-GC/MS (single quad)	s-PLS-DA	9 VOCs/_/100%/80%
[54]	Malaria[b]	A) 17 febrile subjects with malaria infection vs. 18 febrile subjects without malaria infection	3–15	Yes/not/not	Late expiratory breath/SamplePro Flexflim sample bag (3L)	Mass spectrometry -Off-line breath analysis: TD (Tenax® 60/80, Carbograph 1 60/80 & Carboxen 1003 40/60)-GC/ MS (TOF)	Cumulative abundance metric and nearest mean classification algorithm (binary classification)	6 VOCs/83%/71%/94%
							Student's *t*-test	1 VOC/69%/_/_
								1 VOC/77%/_/_
[55]	NAFLD[c]	A) 37 obese with NAFLD vs. 20 obese with normal liver	6–18	Not/yes/not	Late expiratory breath/Mylar® bag	Mass spectrometry-Off-line bag sampling breath analysis: SIFT-MS	CDA & stepwise variable selection	4 ion peaks/AUC = 0.763/_/_
							ANCOVA	5 VOCs/_/_/_
[56]	CLD[c]	A) 49 CLD (20 with avanced fibrosis, 20 without avanced fibrosis & 9 with unavailable pathology) vs. 55 HCTR	CLD: 12.1 ± 3.7 HCTR: 13.6 ± 4.2	Not/yes/not	Late expiratory breath/Mylar® bag	Mass spectrometry-Off-line bag sampling breath analysis: SIFT-MS	ANCOVA	7 VOCs/_/_/_
							Stepwise variable selection, LDA & MLR	5 VOCs/AUC = 0.97/_/_

Table 1. continued

References	Targeted diseases and study population			Breath sampling		Breath analysis	Data analysis	Results of study
	Disease	Sample size/study design	Age (years)	Ambiental air sample/VOCs filter/sampling in the same room	Exhaled breath portion targeted/ breath container	Analytical methods	Statistical methods	No. of VOCs selected/ classification rate/ sensitivity/specificity
[37]	CDK[c]	A) 48 CKD & 8 with a KTx vs. 60 HCTR	4–18	Yes/not/not	End-tidal breath/- -	Mass spectrometry-On-line breath analysis: PTR-TOF-MS	Heat map, PCA & Kruskall–Wallis analysis followed by Dunn's test	6 VOCs/_/_/_
[38]	IBM[c]	A) 62 IBD (11 with UC & 51 with CD) vs. 55 HCTR	IBD: 12.1 ± 3.0 HCTR: 15.7 ± 3.3 CD: 12.4 ± 3.5 UC: 13.2 ± 3.7	Not/yes/not	Late expiratory breath/Mylar® bag	Mass spectrometry-Off-line bag sampling breath analysis: SIFT-MS	LDA, MLR & ANCOVA	3 VOCs/AUC = 0.96/_/_
		B) 51 CD vs. 11 UC						X/_/_/_
[40]	IBM[c]	A) 67 IBD (34 with CD & 33 with UC) vs. 167 CTR (65 gastroenterological CTR & 102 HCTR)	10–17	Yes/not/not	Mixed expiratory breath/Bio-VOC® breath sampler	Mass spectrometry -Off-line breath analysis: IMR-MS	LASSO + LR	18 VOCs/AUC = 0.925/ 95%/69%
		B) 34 CD vs. 33 UC						13 VOCs/AUC = 0.934/ 94%/76%
		C) IBD (34 with CD & 33 with UC) vs. 65 gastroenterological CTR						15 VOCs/AUC = 0.918/ 94%/65%
		D) 67 IBD (34 with CD & 33 with UC) vs. 167 CTR (65 gastroenterological CTR & 102 HCTR) only with directly or indirectly calibrated VOCs						12 VOCs/AUC = 0.888/ 94%/71%
Other technologies								
[41]	Asthma[a]	A) 39 asthmatics vs. 35 HCTR	6–18	Not/not/not	Mixed expiratory breath/Tedlar® bag (3L)	Broadband quantum cascade laser-based spectroscopy	functions of package "limma" (package for data analysis, linear models and differential expression for microarray data)	23 VOCs/_/_/_
	CF[a]	B) 15 CF vs. 35 HCTR						12 VOCs/_/_/_

CTR controls, *HCTR* healthy controls, *CF* cystic fibrosis, *PCD* primary ciliary dyskinesia, *OSAS* obstructive sleep apnea syndrome, *RW* recurrent wheeze, NAFLD nonalcoholic fatty liver disease, *CLD* chronic liver disease, *CKD* chronic kidney disease, *KTx* functional renal transplant, *IBD* inflammatory bowel disease, *UC* ulcerative colitis, *CD* Crohn's disease, *TD* thermal desorption, *GC* gas chromatography, *MS* mass spectrometry, *tof* time-of-flight, *HS-SPME* headspace solid-phase microextration, *GCxGC-tof-MS* comprehensive two-dimensional gas cromatography, *SIFT* selective ion flow, *IMR* ion-molecule reaction, *PTR* proton transfer reaction, *PDMS* polydimethylsiloxane, *DVB* divinylbenzene, *CAR* carboxen, *ANNs* artificial neural networks, *PCR* principal component reduction, *CDA* canonical discriminant analysis, *PCA* principal component analysis, *LR* logistic regression, *PLS-DA* partial least squares-discriminant analysis, *s-PLS-DA* sparse partial least square discriminant analysis, *d-PLS-DA* dissimilarity partial least squares-discriminant analysis, *ASCA* ANOVA simultaneous component analysis, *SLR* sparse logistic regression, *RF* random forest, *SVM* support vector machine analysis, *ANCOVA* analysis of covariance, *LDA* linear discriminant analysis, *MLR* multivariable logistic regression analysis, *LASSO* Least Absolute Shrinkage and Selection Operator, *AUC* area under the curve.

[a]Respiratory diseases.

[b]Infectious diseases.

[c]Metabolic diseases.

[d]It indicates that the identity of the VOCs has not been reported. X indicates that discriminant VOCs or breath print have not been found. Age data presented as range of years, mean of years ± SD or median of years [P25, P75].

ionization—high-resolution MS), or PTR-MS.[24,63–65] Noteworthy, SESI-HRMS is one of the most well-positioned technologies holding much promise for the clinical translation of breath analysis. Very recent progress in standardization procedures using this technology will allow the first interlaboratory comparisons to become a reality, a first mandatory step for it to be introduced into pediatrics routine clinical practice.[24,66,67] A detailed description of on-line breath analysis technologies is out of the scope of this review. A good summary can be found elsewhere.[24]

Data analysis

The first step in-breath VOCs data analysis is the conversion of spectral data to an amenable peak matrix for subsequent statistical analysis. This was hardly ever described in any of the studies included in this qualitative synthesis. In most cases, this relies upon point and click solutions by proprietary-vendor software hampering reproducibility and interlaboratory comparisons. Following this pre-processing step, a plethora of different supervised classifier algorithms were used including either linear (e.g., partial least squares discriminant analysis,[45,46] or non-linear (e.g., random forest, support vector machine[68–70]) models with their predictive performance evaluated in the same development dataset using bootstrapping or any form of cross-validation (n-fold, leave-one-out, etc.) in the majority of cases (Table 1). Three of them divided their population between training and a test set that neither is a real external validation.[42,49,55] However, none included an external validation with a new group of subjects. Notice that volatilome datasets are usually high dimensional (larger number of VOCs or spectral peaks measured compared to the number of subjects in the study), hold multiple correlated variables with a rather low representation of VOCs across all samples (leading to sparse matrices). This raises associated problems such as multicollinearity and the curse of dimensionality among others[71,72] challenging their statistical analysis. Finally, VOCs identification was performed mainly via spectral matching against the NIST (The National Institute of Standards and Technology) library. The use of retention indexes (RI) as calculated using a series of RI standards for identification purposes was solely reported in five cases. None of the reviewed studies reported the level of confidence used for VOCs identification.[73]

Reported exhaled VOCs in pediatric diseases

A comprehensive list of selected discriminant VOCs classified according to chemical families and found to be relevant for any of the studies included in the qualitative synthesis is summarized in Supplementary Table S3. Accordingly, in contrast to the adult population,[18] childhood asthma was demonstrated to be negatively associated with acetone levels.[45,49] Conversely, alkane levels were positively associated with asthma in the pediatric population[17] resulting in line with the adult population. The majority of alkanes detected in breath had been described as by-products of lipid peroxidation a condition usually triggered by inflammation, which is in turn typically associated with asthma.[3] Other products derived from lipid peroxidation such aldehydes[3,74] were dysregulated in childhood asthma,[45–47,49] a trend observed in adults too.[17] Of note, acetone, alkanes, and aldehydes were also reported to be dysregulated in childhood diseases other than asthma, e.g., chronic kidney disease (CKD), nonalcoholic fatty liver disease (NAFLD), inflammatory bowel disease (IBD), or bacterial infection.[37,40,53,55] On the other hand, differences in isoprene (a major component of human breath along with acetone)[1] were reported between obese controls and NAFLD patients[55] and between CKD patients and healthy controls.[37] Finally, elevated levels of ammonia were found in the exhaled breath of subjects with liver, bowel or kidney function problems.[37,40,55] Altogether, results in Supplementary Table S3 suggest low diagnostic specificity for most of the reported breath VOCs when they are individually considered as single biomarker diagnosis. Notice for example the case of 2-butanone that has been associated with asthma, *S. aureus* infection in CF patients, and IBD. However, each specific pathology is rather linked to a particular combination of breath VOCs, a pattern that constitutes the entire breath test.[75] In this regard, reported exhaled VOCs patterns for asthma and CF and for asthma exacerbations studied in the pediatric population are depicted in Fig. 2. Notice that despite sharing some commonalities, a totally different pattern arises from asthma and CF. The utility of highlighted breath-based diagnosis markers without an understanding of the biochemical mechanisms involved in their production has been previously questioned.[76] These biochemical mechanisms can be studied through functional analysis. However, one should consider the rather low metabolism coverage that can be attained based solely on VOCs to a correct interpretation of such functional analysis outcomes. To further explore the fraction of the human metabolic network covered by breath VOCs analysis in pediatric diseases we performed a network-based pathway enrichment analysis based on FELLA[32] using compounds in Supplementary Table S3 as input data. Based on this, FELLA builds a hierarchical representation of human metabolism using the Kyoto Encyclopedia of Genes and Genomes (KEGG) as database[77,78] and applies a null diffusive process deriving a relevant sub-network (Supplementary Fig. S1) with a list of affected pathways and intermediate entities (enzymes and reactions).

Quality assessment

Results for the quality assessment of the selected studies through the QUADAS-2 scoring system are summarized in Fig. 3. QUADAS-2 analysis showed concerns across several domains with index test and patient selection accounting for the highest risk of bias (Fig. 3a, b) and the two reviewers showing a good agreement ($k =$ 0.81 (IC 95% 0.75–0.88)) (Supplementary Tables S4 and S5). The most important source of bias was related to the analysis of VOCs (index test) since none of the selected studies went through an external validation using new independent cohorts. Those studies using gas sensor arrays were considered to be at high risk of bias because VOCs identification is intrinsically prevented using such technologies.[36,39,42,43] On the other hand, patient selection was judged to be at high risk of bias for almost all cases as well. This is because the majority of studies consisted of case–control designs. Thus, diagnostically challenging cases were excluded leading to overoptimistic results and to a flattering impression of VOCs diagnostic performance. Nevertheless, a more realistic patient selection strategy was used in studies involving ADEM,[48,49,79] RASTER,[30,51] EUROPA,[42] and FLAME[50] cohorts and in Neerincx et al.[53]. On the other hand, Benedek et al.,[43] Schaber et al.[54] and Alkhouri et al.[55] included patients with favorable conditions to develop the targeted pathology as a control instead of considering healthy controls. Moreover, Monasta et al.[40] used both gastroenterological controls and healthy controls. The flow and timing domain were considered at high risk of bias too. This was because in case–control designs the healthy controls were not receiving the same reference standard as the case subjects. In contrast, the time interval between the reference standard and the index test was appropriate due to the own chronic nature of the diseases considered in many studies. Finally, the reference standard domain did not introduce concern at the exception of two studies where the authors themselves questioned the methodology for disease diagnosis: Alkhouri et al.[55] using a conventional hepatic ultrasound instead of a biopsy for NAFLD diagnosis and van de Kant et al.[48] considering just two episodes recorded on ISAAC (International Study of Asthma and Allergy in Childhood) questionnaire to diagnose recurrent wheeze. Finally, the concern regarding the applicability of the identified studies to our review question was generally low (Fig. 3c). There was just a single study where the applicability concerning the reference standard was unclear since it dealt with recurrent wheezing, which is defined as a symptom rather than a disease.[48]

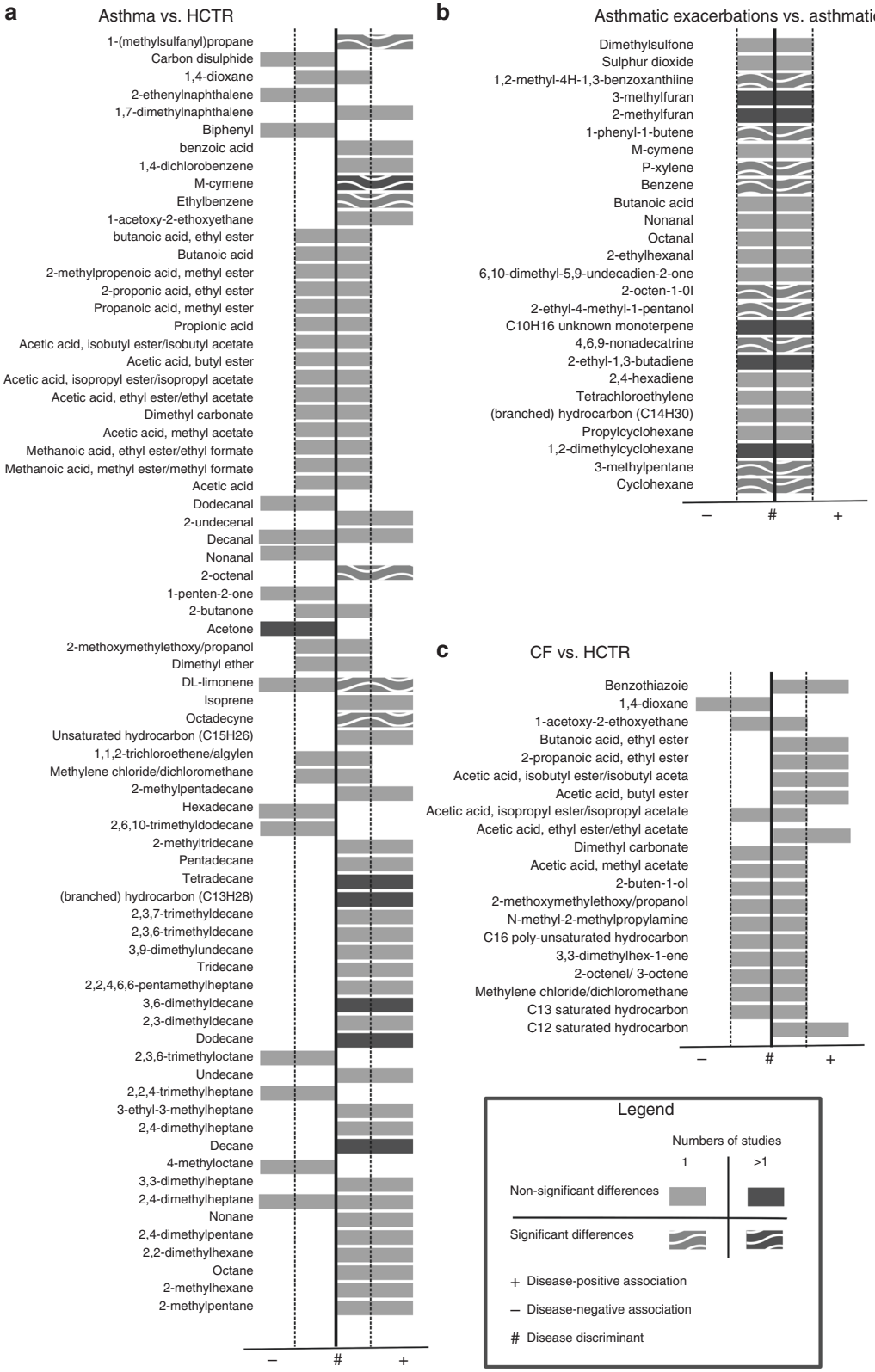

Fig. 2 VOCs discriminant profile summarized across the studies included in the qualitative synthesis. a Asthma or allergic asthma patients vs. healthy controls (HCTR). **b** Asthma with exacerbations vs. stable patients. **c** Cystic fibrosis (CF) vs. HCTR.

DISCUSSION

Breath exhaled VOCs for clinical pediatrics: are we there yet?

Despite we found a substantial body of evidence, it was judged to be at high risk of bias proving that exhaled VOCs profiling is unlikely to reach pediatrics clinical practice in its current state. All the studies included in this qualitative synthesis remain stagnant in a very preliminary preclinical discovery phase with most of them seeking to agnostically screen VOCs that can be further

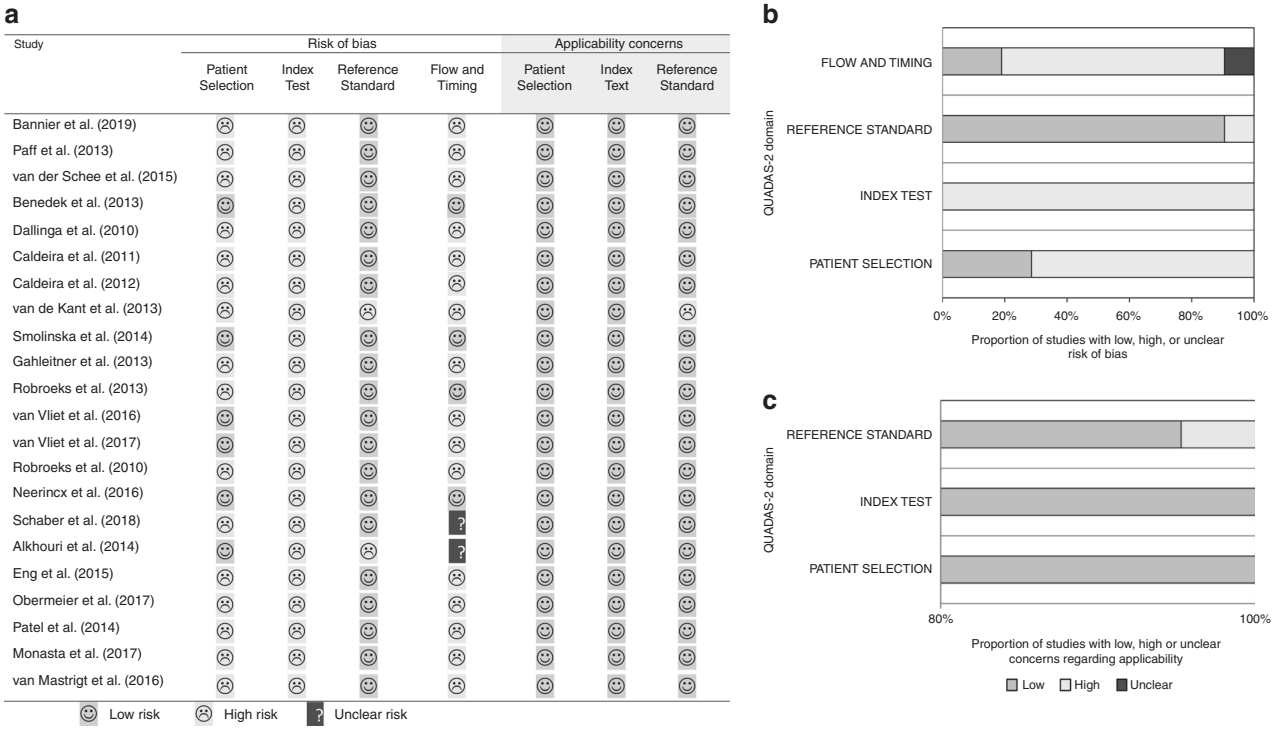

Fig. 3 Quality assessment of the included studies in the qualitative synthesis through QUADAS-2 scoring system. a Summary for individual studies. Display of **b** risk of bias and **c** applicability concerns across different domains.

associated with different disease status. This discovery phase lies far ahead of the analytical method validation and eventually any clinical validation accepted by the medical community. Noteworthy, if a breath VOCs-based test is to reach clinical practice, it should undergo a three-phase process including the discovery, test validation, and evaluation for clinical utility phases.[80,81] Importantly, one needs to face the discovery/exploratory and test validation phases having in mind the checklist criteria that needs to be addressed to determine the readiness of an -omics test for use in a prospective clinical trial.[82] The discovery phase should ideally be regarded as part of the process aimed at assessing whether the VOCs-based test has a reasonable chance to demonstrate clinical validity. Hence, full completion of this discovery phase involves the following four steps: (i) data quality control; (ii) computational model development and internal validation; (iii) external validation; and (iv) release of data, code, and the fully specified computational procedures to the scientific community.[80] Of mention, none of the studies in this qualitative synthesis include these four steps. In consequence, their results will hardly translate into clinical practice.

Breath exhaled VOCs for clinical pediatrics: the path to the clinics
Designing exploratory experiments in compliance with the aforementioned four mandatory steps should push forward the breath VOCs clinical translation research. In this regard, the breath community should draw on the metabolomics community from which it shares multiple communalities. Adopting well-established metabolomics best practices in the discovery phase would ensure a smooth transition towards the test validation phase paving the way to an eventual clinical translation. In this regard, the breath community should firstly agree in a clear set of guidelines and minimum reporting standards as the metabolomics community had done so far.[73,83,84] This would impact, for example, the way that VOCs identification are being reported to date and would promote adding their confidence identification levels, increasing transparency. Notice that these levels are not reported in any of the reviewed studies. Reporting VOCs identifiers other than their name is also recommended. Relying just on names might introduce ambiguity, particularly if trivial instead of systematic names are used. Consider for example the compound reported as *1-penten-2-on* by Dallinga et al.,[44] a study included in our systematic review. With any further identification, assigning this name to a structure is impossible since the suffix *–on* is not indicative of a functional group. The most straightforward assumption is that this might be a typo but with no further information this is just an assumption. This would have been prevented if other identifiers had been considered. In this regard, reproducible VOCs identifiers such as the International Chemical Identifier (InChI), or the Simplified Molecular-Input Line-Entry System (SMILES) have a key role in accessing compound data and they can ensure cross-referencing across studies and databases such as the HMDB.[85]

Consistent quality assurance along with a breath VOCs study should be regularly checked to ensure the quality of the measured data. This is usually done in metabolomics through regular testing of the analytical performance of the instrument and using quality control samples that account for sources of variability non-related to the biological phenomena under study. Quality control samples should ideally consist of pooled samples entering the study, which are regularly measured through the study sequence. Preparing such pooled quality control samples is straightforward in metabolomics but it might result in intricately complicated for VOCs breath analysis. A turn around would be using retention indexes external standards (e.g., *n*-alkanes) with a twofold objective: accounting for retention time deviations as they were originally intended to and to estimate instrument variability along this sequence. On the other hand, the quality of data is largely impacted by MS-data pre-processing procedures (centrodization, denoising, baseline correction, alignment, and peak picking) a step that is overlooked in most of the studies included in our qualitative synthesis. Neither external validation nor data analysis transparency is contemplated in any of the reviewed studies. The

lack of external validation is the main attributable cause explaining why breath VOCs research in the pediatrics population remains stagnant in a preliminary exploratory phase. In the absence of an external validation step, the risk of overfitting rises, and results should be carefully interpreted since they might likely overestimate real findings. Moreover, the lack of transparency in data analysis is also an issue. Both need to be urgently addressed if breath VOCs analysis is intended for further clinical development. Reproducible and transparent data analysis involves making data and metadata available through specialized repositories that can be publicly accessed. This should be nowadays feasible using for example Metabolomics Workbench or Metabolights[86] to name just a few of them. Of note, data should be made available in community-accepted standard open formats. In the case of mass spectrometry data, this would be for example the vendor-neutral standard mzML format.[87] In addition, transparent data analysis involves making reproducible computational procedures and its associated code available as well. Using open-source solutions based on scripting languages (R, Python, Matlab) for data analysis is a good avenue to easily maintain computational code fully reproducible. In addition, hosting such code in platforms for collaborative software development such as GitHub allows fully sustainable access to it.

The outcome of a properly designed discovery phase should ideally be a panel of properly identified breath VOCs putative markers. The step that follows to turn them into clinically reliable markers is the test validation phase. In this validation phase, an analytical method directed towards the detection of the panel of exhaled VOCs markers is optimized[88] allowing to lock down most of the analytical aspects of breath VOCs analysis. Then, the assay performance of this targeted method can be computed using established analytical metrics such as accuracy, precision, coefficient of variation, sensitivity, specificity, linear range, limit of detection, and limit of quantification, as applicable. It is only at this stage where the much-discussed standardization needs[76] makes sense and can be reliably applied. In fact, there is an old debate in-breath community on the need to standardize breath VOCs sampling and analysis.[76] This lack of standardization has long been blamed for no progression from laboratory to the routine clinical setting. Despite we agree on the need for standardization, we consider that it needs to be redefined and clarified. In fact, trying to standardize the discovery phase of breath VOCs studies could prove counterproductive and too restrictive. At this stage, VOCs profile composition is yet to be characterized and therefore breath is profiled in a chemically unselective manner. Thus, large room for experimental maneuver is needed and a combination of different sampling and analysis methods is commonly used so as to ensure VOCs coverage. This does not exempt to meet minimum reporting standards regarding experimental conditions, data analysis, and breath VOCs identification. In fact, these reporting standards are pivotal for further method validation and an eventual clinical validation of the VOCs breath test.

Limitations

Our search strategy was deliberately broad and inclusive to capture all studies using breath VOCs analysis in the pediatric population regardless of the study design, measurement technology, and disease conditions. This strategy might negatively impact the risk of bias in some domains. Meta-analysis was deemed unfeasible because of the high heterogeneity of the studies included in the qualitative synthesis. This heterogeneity was caused by: (i) deliberate consideration of totally different diseases; (ii) inconsistent definition of the control groups across different studies (healthy controls, stable patients, subjects presenting certain symptoms, etc.); (iii) use of different technologies (gas sensor arrays, GC/MS); (iv) inconsistent panel of differential VOCs across studies included in the qualitative synthesis. In addition, the number of published studies including breath VOCs analysis in the pediatric population is relatively low, limiting the comparison for some diseases.

CONCLUSIONS AND PERSPECTIVE

We present the first systematic review of the use of exhaled VOCs analysis in clinical pediatrics. We conclude that the much-heralded clinical potentiality of breath volatilome analysis in clinical pediatrics is yet to be formally demonstrated. Our descriptive synthesis of the evidence shows a substantial number of studies using volatilome analysis in the pediatric population during the last 10 years. Despite this, conclusions about breath VOCs clinical potentiality remain elusive. This is not only attributed to the heterogeneity of studies hampering pooling of data for a proper meta-analysis but also to the fact that all reviewed studies remain stagnant in a very preliminary exploratory phase with no further progression. At this phase, exhaled VOCs tests are usually far from being a mature diagnostic tool and therefore claiming their clinical potential is too premature. Our view is that improved experimental designs of such exploratory studies adopting well-established metabolomics best practices such as reporting minimum standards for VOCs identification, using disclosing code, and experimental data through public repositories should help to push forward exhaled breath VOCs research paving the way to an eventual clinical translation.

ACKNOWLEDGEMENTS

Rosa A. Sola Martínez acknowledges her fellowship from the Spanish Ministry of Science, Innovation, and Universities (FPU18/00545). This work was supported by grants from the Instituto de Salud Carlos III through the project "PIE15/00051" (Co-funded by European Regional Development Fund/European Social Fund "A way to make Europe"/"Investing in your future"), the Ministry of Science, Innovation, and Universities (MCIU), the State Research Agency (AEI) and the European Regional Development Fund (FEDER), RTI2018-094393-B-C21-MCIU/AEI/FEDER, UE, and the Seneca Foundation CARM, 20786/PI/18.

AUTHOR CONTRIBUTIONS

R.A.S.M.: Conceptualization and design, methodology, acquisition of data, data curation, formal analysis, and writing-original draft. J.M.P.H.: Conceptualization and design, methodology, acquisition of data, and writing-review. Ó.Y.T.: Data curation, formal analysis, writing-review, and editing. M.C.D.: Writing-review and editing, project administration, and funding acquisition. T.d.D.P.: Conceptualization and design, methodology, data curation, formal analysis, writing-review and editing, supervision, project administration, and funding acquisition. M.V.C.: Conceptualization and design, methodology, data curation, formal analysis, writing-review, and editing. All authors read and approved the final.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41390-020-01116-8) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

Patient consent: Participant consent was not required for this research.

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