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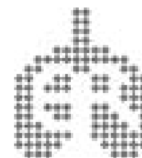

REVIEW

Bronchoalveolar lavage, sputum and exhaled clinically relevant inflammatory markers: values in healthy adults

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ABSTRACT: Bronchoalveolar lavage (BAL), induced sputum and exhaled breath markers (exhaled nitric oxide and exhaled breath condensate) can each provide biological insights into the pathogenesis of respiratory disorders. Some of their biomarkers are also employed in the clinical management of patients with various respiratory diseases. In the clinical context, however, defining normal values and cut-off points is crucial. The aim of the present review is to investigate to what extent the issue of defining normal values in healthy adults has been pursued for the biomarkers with clinical value.

The current authors reviewed data from literature that specifically addressed the issue of normal values from healthy adults for the four methodologies.

Most studies have been performed for BAL (n=9), sputum (n=3) and nitric oxide (n=3). There are no published studies for breath condensate, none of whose markers yet has clinical value. In healthy adult nonsmokers the cut-off points (mean+2SD) for biomarkers with clinical value were as follows. BAL: 16.7% lymphocytes, 2.3% neutrophils and 1.9% eosinophils; sputum: $7.7 \times 10^6 \cdot mL^{-1}$ total cell count and 2.2% eosinophils; nitric oxide: 20.2 ppb.

The methodologies differ concerning the quantity and characteristics of available reference data. Studies focusing on obtaining reference values from healthy individuals are still required, more evidently for the new, noninvasive methodologies.

KEYWORDS: **Airway inflammation, bronchoalveolar lavage, exhaled breath condensate, induced sputum, nitric oxide, normal values**

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Bronchoalveolar lavage (BAL), induced sputum, the determination of fractional exhaled nitric oxide (*F*eNO) and the measurements of many compounds in exhaled breath condensate (EBC) represent the most important methodologies that can give relevant biological insight to many respiratory disorders. BAL is performed by instilling, and recovering by gentle suction, aliquots of sterile saline into the sub-segmental bronchi through a fibreoptic bronchoscope with the aim of analysing cells and solutes from the lower respiratory tract. Induced sputum is performed by inducing expectoration through the inhalation of hypertonic saline, with the aim of analysing cells and solutes considered representative of larger airways. *F*eNO and EBC share the concept of detecting one (nitric oxide; NO) or more (in EBC) chemical compounds from exhaled air, which can function as useful biomarkers providing information on the inflammatory and oxidative pathways in the airways and respiratory system. Despite differences in the methodology for obtaining the biological sample (invasive *versus* noninvasive), the nature of the sample to be analysed (liquid *versus* breath air), the detected biological markers useful in clinical practice (cellular markers for BAL and induced sputum *versus* exhaled molecular marker), and even the facilities, equipment and time needed to obtain the relevant data (table 1), these ''biological investigations'' share a number of features. The basic principle underlying the analysis of the data on lung and airway biology is to provide additional information to those derived from other more traditional diagnostic sources (*e.g.* imaging, lung function).

TABLE 1 Clinical applications of bronchoalveolar lavage, induced sputum, fractional exhaled nifric oxide and exhaled breath condensate in inflammatory disorders

	Bronchoalveolar lavage	Induced sputum	Fractional exhaled nitric oxide	Exhaled breath condensate
Standardisation	ERS [1–3]	ERS [4]	ATS/ERS [5]	ATS/ERS [6]
Reproducibility and validity	Reproducibility assessed and methodology validated in normal subjects and disease status (mainly ILD)	Reproducibility assessed and methodology validated in normal subjects and disease status (mainly asthma, COPD)	Reproducibility assessed and methodology validated in normal subjects and asthma	Data available for some mediators
Nature of sample	Biological fluid containing cells and acellular components	Biological fluid containing cells and acellular components	Exhaled air containing NO gas	Biological fluid containing acellular components
Biomarker(s)	Differential cell count, specialised markers[¶]	Total and differential cell count specialised markers[¶]	NO ppb	pH, various mediators
Feasibility: facilities and equipment needed	Thoracic endoscopy, basic cellular lab, outsourcing for specialised analyses	Pulmonary function lab, ultrasonic aerosol, basic cellular lab, outsourcing for specialised analyses	NO analyser	EBC condenser, pH analyser, HPLC or MS
Time for evaluation	1 working day[+]	1 working day[+]	Online	Online[§], days to weeks
Main field of application[#]	ILD, exposure to toxic substances	All airway diseases, ILD, exposure to toxic substances	Asthma and allergic diseases	COPD, asthma allergy, infections, exposure to toxic substances
Clinical applications	In the diagnostic process of sarcoidosis, IPF and other ILDs [7–9]	As an additional method to manage asthma [10–15]	As an additional method to manage asthma. [15–20]	NA

ERS: European Respiratory Society; ATS: American Thoracic Society; ILD: interstitial lung diseases; COPD: chronic obstructive pulmonary disease; NO: nitric oxide; EBC: exhaled breath condensate; HPLC: high performance liquid chromatography; MS: mass spectrometry; IPF: idiopathic pulmonary fibrosis; NA: not available. [#]: only applications related to inflammatory markers in lung and airway diseases of adult patients are reported; [¶]: specialised markers can be diagnostic in bronchoalveolar lavage (*e.g.* CD1+ cells in histiocytosis X, mineralogic assays for assessing exposure) and in sputum (*e.g.* asbestos bodies); [+]: 90 mins is usually sufficient for an expert team to obtain the results, 1 working day is intended for a report in clinical practice; [§]: online evaluation in EBC is only for pH and H_2O_2 determination.

Each technique followed a similar development pathway, from the first description in research centres [21–24], to procedure standardisation, usually with the aid of *ad hoc* collaborative groups or Task Forces sponsored by scientific societies [1–6], with the aim of providing guidance for the reproducibility of the results obtained. From an investigational point of view, all techniques have been widely applied to more or less all disease conditions in order to gain information on the pathogenesis of and, ultimately, improve the ability to diagnose and treat respiratory disorders. From a clinical point of view, each technique has been proposed in selected clinical scenarios [7, 10–14, 16–19]. The clinical application of one of the BAL biomarkers, *i.e.* cell differential count, is now widely accepted and recommended in clinical guidelines as a diagnostic tool for sarcoidosis and idiopathic pulmonary fibrosis [8, 9], while other biomarkers, induced sputum cell counts and *F*eNO values are considered useful additional tools in the management of asthma [6, 7, 10–20]. In contrast, the determination of molecular markers in EBC is still awaiting an accepted clinical application. In this context, the collection of data from healthy subjects to use as benchmark reference values is mandatory.

Aim of the literature review

The aim of the current review is to investigate to what extent the issue of defining normal values in healthy adults has been pursued for BAL, induced sputum and exhaled biomarkers. For each methodology the current authors reviewed papers that: 1) specifically addressed the problem of normal reference values, *i.e.* disease-focused studies featuring matched control populations were not included; 2) comprised populations of healthy adult subjects; and 3) considered the clinically useful inflammatory biomarkers, *i.e.* those used in clinical practice as an aid in the management of respiratory patients. The authors also included the analysis of such data for EBC, a promising tool to identify new, noninvasive biomarkers.

Search strategy

In order to accomplish the task, a systematic literature search was performed as follows. Pertinent studies were retrieved using online databases for medical literature (PubMed and HighWire Press). Reference lists from published articles or reviews were also used, and only papers published in English were considered. The search was further limited to studies that enrolled >30 healthy study subjects, since below this threshold data was considered to come from underpowered protocols. Five reviewers (B. Balbi, P. Pignatti, M. Malerba, M. Corradi and P. Baiardi) independently examined the titles and abstracts of all identified papers to confirm fulfilment of inclusion criteria. They recorded the papers' characteristics independently using a pre-designed data abstraction form.

BAL

In 1974, the first paper detailing BAL dealt with normal values, as the authors selected normal subjects and patients undergoing fibreoptic bronchoscopy (FOB) for ''evaluation of intrathoracic lesions'' [21]. BAL is a standardised methodology. Its reproducibility has been shown both in comparing serial BAL data obtained by repeating the procedure up to five times with an interval of ⩾6 weeks in the same subjects [25] and in

comparing the results obtained in different centres [26]. This particular study showed high (*i.e.* >0.8) correlation coefficients for all cell types. It should also be considered that repeated or serial BAL is a cause of airway inflammation in itself, not limited to the site of first lavage and characterised by increased proportions of inflammatory cells in BAL (mainly neutrophils). These changes last ⩾72 h. [27]. The fluid recovered after infusion of the first aliquot of BAL has, in some papers, been defined as ''bronchial lavage'', which suggests that it might represent the airway inflammation present in larger airways better than the later aliquots, which are supposed to better reflect the smaller airways. This assumption, however, never reached an accepted standardisation [3].

Over the years BAL has been used to investigate inflammatory parameters of the lower respiratory tract, particularly in the field of interstitial lung diseases (ILD) but also in many other conditions, such as infection, neoplasms, exposure to toxic substances, asthma and chronic obstructive pulmonary disease (COPD) [2]. In addition, from a clinical point of view it has been widely applied to many disease conditions. Apart from its use in infections (*e.g.* ventilator-associated pneumonia) or in the diagnosis of peripheral cancers, BAL has acquired an accepted clinical role in the field of inflammatory biomarkers useful in the diagnostic process of ILD [8, 9]. In addition, it can be diagnostic *per se* in a limited number of rare disorders, *e.g.* increased BAL proportions of CD1+ cells for histiocytosis X (table 1).

Results of the literature search

Focusing on studies on normal values, nine studies specifically designed to provide data on BAL cellular normal reference values in adult subjects, fulfilling the current literature search criteria, have been published as observational studies (table 2) [26, 28–35]. Eight of these were performed in North America and only one in Europe [35]. All except one were mono-institutional. Seven were approved by an Ethical Committee and informed consent was obtained from participants in one further study. Overall, 760 subjects participated in these studies and underwent FOB and BAL in order to provide BAL reference data. Most of the subjects were male, nonsmokers and aged <50 yrs. Only four studies specifically evaluated the influence of age on BAL characteristics [26, 33–35], and most of the subjects concerned were nonsmokers. Since the vast majority of the studies were from North America, in particular the USA, it is not surprising that in some cases volunteers were reimbursed for participation. The type and number of clinical and instrumental parameters needed to define normal subjects (*i.e.* the inclusion criteria) varied greatly, from basically no specification to a complex and costly assessment including chest radiographs, medical history, physical examination, blood count and spirometry or even carbon monoxide diffusing capacity measurements. Three studies mentioned the history of allergies as an exclusion criterion.

The number of subjects enrolled in each study varied 34–191. The total fluid infused ranged 100–300 mL, divided into 3–10 aliquots, reflecting the high variability of accepted methodologies in performing BAL [3]. Usually BAL was performed in a sub-segment of the middle lobe or lingula.

One study excluded the first aliquot from analysis [35]. The majority of studies employed cyto-centrifuge to obtain cell differential counts, *i.e.* the method most used nowadays (table 3). Despite all these inconsistencies, the data are quite consistent in defining the characteristics of normal reference BAL at least for the differential cell count, *i.e.* the only BAL biomarker with a clinical value in the differential diagnosis of ILD. The majority of data reviewed dealt with healthy young to middle-aged nonsmoker subjects. In these subjects the upper cut-off points (*i.e.* mean+2SD) for lymphocytes, neutrophils and eosinophils were 16.7, 2.3 and 1.9%, respectively (table 3). Compared with nonsmokers, asymptomatic smokers have an increased proportion of macrophages, lower percentages of lymphocytes and usually increased percentages of neutrophils and/or eosinophils. The limited data available on the influence of age on BAL cell composition appear to indicate an increased proportion of neutrophils in older subjects, a finding in line with similar observations in induced sputum.

Acellular components of BAL

The numerous acellular components measured in BAL supernatants only have value as investigative tools, owing largely to the unsolved issue of the dilution factor [3]. This problem was first approached using internal markers such as urea, but in the latest European Respiratory Society (ERS) Guidelines it is recommended that acellular markers be expressed in terms of their concentration per unit of supernatant [3]. A list of normal values is provided in the relative guidelines. The current authors did not include this type of biomarker in the analysis since the search criteria only focused on clinically relevant biomarkers. This exclusion does not apply to those markers defined as ''specialised markers'' *i.e.* identifying in themselves the presence of a certain condition or exposure to some inorganic material (*e.g.* asbestos bodies as exposure to asbestos; table 1).

Problems open for discussion

The influence of age and smoking history

In spite of the great volume of data collected on BAL reference values, most deal with young or middle-aged subjects among whom smokers are a minority. Thus, the data available as normal reference values for asymptomatic smokers aged >50 yrs are rather limited.

INDUCED SPUTUM

Although spontaneous sputum analysis has been used for many years for microbiological and cytological diagnoses, the presence of large quantities of dead cells in spontaneous sputum and the inability of many subjects to expectorate spontaneously prompted the use of induced sputum as a method for assessing airway inflammation, a methodology first described in 1978 [22]. Thereafter, induced sputum analysis was applied to evaluate airway inflammation in airway diseases, in particular asthma and more recently COPD, but also cystic fibrosis (CF) and other diseases with pulmonary involvement such as ILD, pneumoconiosis, infections in the immunocompetent and immunocompromised host and cancer [4, 12–14]. While the method has proven to be reasonably safe, it can also *per se* induce transitory changes in the composition of the cellular components of sputum after 24 h in healthy subjects, with an influx of neutrophils into the airway lumen detected by induced sputum analysis [36]. International Guidelines on sputum [4] provide guidance on

TABLE 2 Characteristics of focused on bronchoalveolar lavage in healthy adults

First author [Ref.]	Subjects n	Inclusion criteria	Exclusion criteria	Age yrs	Smoking history	Sex
WARR [28]	36	H	NA	(NA) 23±1	24 NS, 12 S	NA
LAVIOLETTE [29]	42	H, PFT, $D_{L,CO}$	Current smoking	M: (19–32) 23.9 F: (20–41) 25.7	38 NS, 4 FS	22 M, 20 F
ETTENSOHN [30]	78	CXR, PFT, PE	History of PD, current smoking, Med, VI	(20–36) 26.3	NS	44 M, 34 F
Anon. [26]	191	CBC, CXR, PFT	NA	(19–72) 42.8±0.9	77 NS, 50 FS, 64 S	106 M, 85 F
MERCHANT [31]	130	NA	History of RD, VI	NS: (20–48) 30 S: NA	111 NS, 19 S	NS 70 M, 41 F, S NA
EVERSON [32]	163	H	History of RS, allergies	(18–35) NA	138 NS, 25 S	112 M, 49 F, 2 NA
MEYER [33]	39	H, PFT	History of smoking, allergies, RD, Dust	Group I: (20–36) 28.1±1.3 Group II: (45–55) 50.8±1.3 Group III: (>65) 69.3±1.0	NS	17 M, 22 F
MEYER [34]	34	H, PFT	History of smoking, allergies, RD, Dust	Group I: (19–36) 27±1 Group II: (64–83) 71±1	NS	19 M, 15 F
EKBERG-JANSSON [35]	47	NA	AD, SDTh, HF, ASD, Inf, Med	60	30 S, 17 NS	47 M

Data are presented as n, (range) mean or (range) mean±SEM. Anon.: anonymous; H: healthy; NA: not available; PFT: pulmonary function tests; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide; M: males; F: female; NS: never-smoker; FS: former smokers; CXR: chest radiograph; PE: physical examination; PD: pulmonary diseases; Med: medication at the time of the study; VI: viral or other illness; CBC: complete blood counts; S: current-smoker; RD: respiratory disease; RS: respiratory symptoms; dust: history of exposure to dust; AD: airways diseases; SDTh: scoliosis or deformation of thorax; HF: heart failure history; ASD: any severe disease; Inf: infections.

the most appropriate methods of induction and analysis of sputum cells and soluble mediators. The methodology has been demonstrated to be repeatable, with a high intraclass correlation coefficient (ICC) at least for the percentages of eosinophils (0.94), neutrophils (0.81), macrophages (0.71) and metachromatic cells (0.70), while total cell counts and lymphocytes had low ICC values [37]. The method of sputum processing can influence the results obtained, depending on whether the sample is processed as selected portions or ''*in toto*'' (presence of high numbers of squamous cells) and on whether or not the material is solubilised with dithiothreitol [38]. Furthermore, as for BAL, sputum should also be processed as soon as possible or maintained at 4°C for no more than a few hours.

Induced sputum is thus validated in normal subjects and in disease conditions, such as asthma and COPD, and eosinophil count is now considered as a useful additional test in the management of asthmatic patients (table 1) [12–15, 39, 40].

Acellular components of induced sputum

Many studies have addressed the evaluation of soluble markers in induced sputum: cytokines, chemokines, eicosanoids, markers of oxidative stress and others. Thus, potentially, many compounds may have not only research relevance but also clinical relevance, providing an additional parameter for diagnosis or a reliable biomarker for the assessment of response to therapy. Unfortunately, however, International Guidelines state that the validity of these measurements as clinical tools remains uncertain, and here (as in the case of acellular BAL components) the issue of undefined dilution factor in supernatant of induced sputum is certainly relevant [4]. As with BAL, induced sputum may also demonstrate the presence of certain ''specialised markers'', *i.e.* those markers identifying a certain condition or exposure to some inorganic material in themselves (table 1).

Results of the literature search

Overall the current authors found only three studies primarily focused on normal values in healthy adult subjects fulfilling the present literature search criteria. All were mono-institutional and the number of subjects involved was much lower than for BAL. In fact, until 2000 no study was available for sputum cell count in healthy controls, even though several studies had previously been published with small numbers of healthy controls as a reference group. The studies of BELDA *et al.* [41] and SPANEVELLO *et al.* [42] were published almost contemporarily reporting data on sputum cell count in 118 and 114 normal volunteers, respectively (table 4). Both studies enrolled nonsmoking subjects with no history of asthma or other respiratory symptoms, no bronchial hyperreactivity or referred symptoms of airway infections in the months prior to sputum induction, and with normal pulmonary function. In the study by BELDA *et al.* [41], 39% of the enrolled subjects were atopic but not exposed to the sensitising agent in the week preceding sputum induction, whereas all the volunteers in the study by SPANEVELLO *et al.* [42] were nonatopic. Sputum was induced with different methodologies: 1) by increasing percentages of hypertonic saline solution, 3, 4 and 5% each for 7 min [41]; or 2) using 4.5% hypertonic saline solution nebulised for periods of progressively increasing length (1, 2, 4, 8, and 16 min) [42]. The percentages of unsuccessful production of sputum were 19.0 and 11.4%, respectively, in the two studies. Sputum selected opaque and dense portions were

TABLE 3 Bronchoalveolar lavage (BAL) values from healthy adults: methodology and cellular data

First author [Ref.]	Aliquots × mL	Site of BAL	Laboratory method	Macrophages %	Lymphocytes %	Neutrophils %	Eosinophils %
WARR [28]#	NA	NA	MS-WG	NS: 78.8±3.8 S 87.0±1.8	NS: 16.7±3.0 S: 6.5±1.1	NS: 4.6±0.4 S: 6.6±0.6	
LAVIOLETTE [29]	10×30	RML	C-WG	NS+FS: 88.6±7.9	NS+FS: 9.6±7.7	NS+FS: 1.7±1.2	NA
ETTENSOHN [30]	3×40	LL	C-WG	NS: 95.1±2.9 NS: M 94.9±3.2 NS: F 95.5±2.4	NS: all 3.9±2.4 NS: M 4.2±2.7 NS: F 3.7±1.9	NS: all 0.7±0.8 NS: M 0.8±0.8 NS: F 0.7 ±0.8	NS: all 0.17±0.9 NS: M 0.2±1 NS: F 0.1±0.5
Anon. [26]#	4×60	RML or LL	C-DQ	NS: 85±1 FS: 86±1 S: 92±1	NS: 11±1 FS: 11±1 S: 5±0.9	NS: 1.6±0.07 FS: 2.1±0.5 S: 1.6±0.2	NS: 0.2± 0.06 FS: 0.5±0.2 S: 0.6±0.1
MERCHANT [31]	5×20/LL/	LL	C-WG	NS: 93.2±5.8 S: 98.6±2.6	NS 6.1±5.6 S 2.16±2.5	NS: 0.5±0.8 S: 0.8±0.9	NS: 0.1±0.4 S: 0.2±0.5
EVERSON [32]#	6×50	RML	C-L	NS+S: 88.3±0.6	NS+S 9.0±0.5	NS+S: 0.9±0.1	NS+S: 0.15±0.02
MEYER [33]#	4×40	RML	C-DQ	NS age group I: 85.8±2.0 NS age group II: 87.5±1.6 NS age group III: 81.5±2.7	NS age group I 12.4±1.7 NS age group II 10.3±1.8 NS age group III 13.2±2.6	NS age group I: 1.84±0.26 NS age group II: 2.13±0.63 NS age group III: 4.61±1.23	NS age group I: 0.11±0.08 NS age group II: 0.07±0.07 NS age group III: 0.71±0.38
MEYER [34]#	4×60	RML	C-DQ	NS age group I: 90±1 NS age group II: 80±3	NS age group I: 8.3±0.9 NS age group II: 17.0±3.3	NS age group I: 1.2±0.2 NS age group II: 2.7±0.7	NS age group I: 0.3±0.1 NS age group II: 0.3±0.1
EKBERG-JANSSON [35]	3×50	RML	C-WG	NS: 87 (75–96) S: 93 (39–99)	NS: 10 (0–23) S: 1 (0–5)	NS: 2 (1–5) S: 2 (0–11)	NS: 0.8 (0–3) S: 0.4 (0–2)

Data are presented as mean±SD or mean (range). #: data presented as mean±SEM. Anon: anonymous; NA: not available; MS: microscope slide; WG: Wright Giemsa staining; NS: never-smoker; S: current smoker; RML: right middle lobe; C: cytocentrifuge; FS: former smoker; LL: left lingula; M: male; F: female; DQ: Diff-Quik staining; L: leukostat staining.

similarly processed with 0.1% dithiothreitol and cell count and viability assessed. Both studies yielded similar results for total cell count and for the percentages of the cells represented in sputum samples, at least for the proportions of eosinophils (table 5). SPANEVELLO *et al.* [42] observed that only sputum macrophages and neutrophils had a normal distribution whereas eosinophils, lymphocytes and epithelial cells did not, suggesting the use of a nonparametric test in the evaluation of these sputum cells. Even if both studies reported total cell count data, neither emphasised the usefulness of these data in the evaluation of airway inflammation. In particular, an increase in the total cell count together with a high percentage of neutrophils immediately suggests a possible airway infection; on the contrary, a high percentage of neutrophils with normal cell distribution points to different causes of neutrophilia.

Whether the age of the subject has any influence on sputum cell distribution is a question that was not addressed in the two studies, since the subjects enrolled were relatively young (mean age 36 and 38 yrs in the studies by BELDA *et al.* [41] and SPANEVELLO *et al.* [42], respectively). In this context, the study by THOMAS *et al.* [43] focused on this point and analysed 66 nonsmoking healthy adults whose age ranged 18–74 yrs. The authors found a significant correlation between age and sputum neutrophils in both male and female subjects, with a notable increase in sputum neutrophils in subjects aged >50 yrs. In fact, the effect of age on sputum cell counts has important implications for the clinical interpretation of the results, reinforcing the notion that control populations should be age-matched, particularly in studies evaluating airway inflammation (*e.g.* patients with COPD) [47].

The data reviewed show that for healthy adult nonsmokers the upper cut-off points (*i.e.* mean+2SD) of the biomarkers with clinical value are $7.7 \times 10^6 \cdot mL^{-1}$ and 2.2% for total cell count and eosinophils, respectively (table 4).

Problems open for discussion

Amount of sputum collected

Healthy controls do not usually produce a large amount of sputum after induction; the International Guidelines for sputum processing [4], as well as other published studies, have left the question open as to whether there should be a weight threshold for the portions collected, below which the analysis loses significance and reproducibility.

Normal upper value of sputum eosinophils

Based on the ERS International Guidelines [4] and other studies [39, 40], a normal upper value of eosinophils of <3 % or <2.5% has been reported. Nonetheless, more recently published studies consider 2% as the cut-off to define sputum eosinophilia. This discrepancy reveals a group of subjects with eosinophils in the range 2–3% who lie in a so called ''no-man's land'' of sputum eosinophilia.

Evaluation of sputum neutrophilia

The normal range of sputum neutrophils still remains a matter for evaluation. Many factors could play a decisive role in determining neutrophil airway inflammation: aging, air pollution, endotoxin contaminant present in the environment,

TABLE 4 Induced sputum values from healthy adults

First author [Ref.]	Subjects n	Inclusion criteria	Exclusion criteria	Age yrs	Sex	Total cells	Macrophages %	Neutrophils %	Eosinophils %	Lymphocytes %	Epithelial cells %
Belda [41]	118#	PFT	NCS A RD AH SC	(18-60) 36	M: 54 F 42	$4.129 \pm 4.81 \times 10^6$ cells·g^{-1}	58.8±21.0	37.5±20.1	0.4±0.9	1.0±1.1	1.6±3.9
Spanevello [42]	114¶	PFT	SAI Atopy A RS AH	(38±13) 38	M: 50 F: 46	$2.7 \pm 2.5 \times 10^6$ cells·mL^{-1}	69.2±13.0	27.3±13.0	0.6±0.8	1.0±1.2	1.5±1.8
Thomas [43]	66	PFT	RS RD AH	(18–74) 44	M: 24 F: 42	$2.1 \pm 2.36 \times 10^6$ cells·mL^{-1}	49.0±25.2	47.0±7.0	0.3±0.6	1.0±1.4	2.5±3.2

Data are presented as (range) mean, (mean±SD) mean, or mean±SD. PFT: pulmonary function tests; NCS: nasal or chest symptoms; A: asthma; RD: respiratory disease; AH: airway hyperresponsiveness; SC: symptoms of cold in the past month; M: male; F: female; SAI: symptoms of airway infections in the 3 months prior to the study; RS: respiratory symptoms; RD: history of respiratory diseases. #: 118 subjects recruited, 96 for sputum analysis; ¶: 114 subjects recruited, 96 for sputum analysis.

TABLE 5 Exhaled nitric oxide fraction values from healthy adults

First author [Ref.]	Subjects	Inclusion criteria	Exclusion criteria	Age yrs	Smoking history	Male/female	Flow rate mL·s^{-1}	Analyser	NO ppb
Olin [44]	2200	Unselected Random general population	NA	(25–75)	NS	1089/1111	50	Niox (Aerocrine, Solna, Sweden)	(2.4–199) 16.0
Haight [45]	48	PFT	Smoking RS, SC, SAI, RD	(18–79) Young group (n=23): 23.4±0.6 Old group (n=25): 71.3±1.1	NS	23/25	50	NOA 208 (Sievers, Seevetal-Hittfeld, Switzerland)	Young group: 25.2±4.8 Old group: 47.8±8.3
Olivieri [46]	204	PFT	Smoking RS, RD, A, allergies	36±9	NS	102/102	50	CLD 88 (EcoMedics, Duernten, Switzerland)	(0.7–28.8) 10.8±4.7

Data are presented as n, (range), (range) median, mean±SD or (range) mean±SD. NO: nitric oxide; ppb: parts per billion; NA: not available; NS: never-smoker; PFT: pulmonary function tests; RS: respiratory symptoms; SC: symptoms of a cold in the past month; SAI: symptoms of airway infections in the 3 months prior to the study; RD: respiratory diseases; A: asthma.

genetic factors, and also bias caused by a nonstandardised procedure of sputum induction [48].

Influence of smoking habit on sputum cell distribution

In spite of the vast number of studies published on the effects of smoking on airway inflammation, a study carried out on a large cohort of smoking and nonsmoking healthy subjects is still lacking.

Influence of sex on sputum cell distribution

BELDA *et al.* [41] found a significant difference in sputum eosinophils between healthy males and females, but they consider this a small difference. Other studies are needed to demonstrate a possible influence of sex on sputum cell distribution.

EXHALED NITRIC OXIDE

The presence of NO in exhaled air was first described in 1991 by GUSTAFFSON *et al.* [23]. Since then, new discoveries have suggested that the measurement of *F*eNO could be a new test for evaluating patients with respiratory diseases, useful in investigating airway inflammation in asthma and other airway diseases, such as COPD or CF, and also for studying other types of respiratory disorders such as ILD, pulmonary hypertension, infections and occupational diseases [49].

There are, however, some important technical factors to be taken into account. First, to measure *F*eNO from lower airways it is important to maintain a consistent expiratory flow rate [50]; faster flow rates decrease *F*eNO concentrations because NO measured at high flow contains a greater proportion of alveolar NO and less bronchial NO. A 50 $mL \cdot s^{-1}$ exhaled flow rate has been recommended [6].

Secondly, since a large amount of NO is produced in the paranasal sinuses [51], it is important to ensure that the soft palate is properly closed prior to sample collection to prevent contamination of lower airway NO with NO from nasal passages. Exhalation against resistance (5–20 cm H_2O) is the preferred method [6, 52].

Thirdly, differences in calibration gases may produce differing results, although analysers from different manufacturers show a sufficient comparability for practical purposes if proper calibration is performed [53]. Fourthly, the influence of ambient NO should be taken into account. Ambient NO must be recorded at each measurement [6], although its effect may be relevant only if it is >35 ppb, an effect that is minimal with the inspiratory filters routinely employed.

Finally, many factors may influence *F*eNO measurements. They include sex and sexual hormones, body weight and age, circadian changes of respiratory function in health and disease, caffeine and alcohol, meals rich in nitrate, genetic background for some enzymes, upper respiratory tract infection, exercise, drugs (including inhibitors of NO synthases) and of course smoking [46, 54–73]. Moreover, *F*eNO levels in healthy subjects are influenced by atopy, *i.e.* a personal or familial tendency to produce immunoglobulin E antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms, such as asthma, rhinoconjunctivitis or eczema dermatitis [74]. Atopy enhances *F*eNO levels even in the absence of allergic symptoms and thus is a relevant confounding factor. It should be considered that NO arises mainly from epithelial cells; while there is a tendency for it to be increased with eosinophilia, it can sometimes be increased in the absence of eosinophilia and may be normal when eosinophilia is present.

Considering all the above issues, the methodology has been demonstrated to be highly reproducible. KHARITONOV *et al.* [75] showed that the measurements of *F*eNO are reproducible (mean $\pm$SD 1.83$\pm$0.75 ppb with a coefficient of variation of 9.5$\pm$4.7%), without diurnal variation and no significant day-to-day variation of measurements, with high feasibility [69] and with an ICC in healthy adults >0.90.

Compared with BAL and induced sputum the evaluation of *F*eNO has the advantage of being totally noninvasive, allowing online evaluation of a biomarker. From a clinical point of view, *F*eNO determination has been mainly applied in allergic airway diseases (table 1) [19, 20]. In this context, *F*eNO is validated for clinical use as an additional parameter to lung function, while none of the other exhaled biomarkers are yet at this stage. It is employed as an additional useful test in the monitoring of asthmatic patients and in assessing the need for changes in treatment regimens (table 1) [15–20].

Results of the literature search

Besides the Task Force recommendations [5], the current authors found three studies primarily focused on normal values in healthy adult subjects fulfilling the literature search criteria, which were all published in 2006 (table 5) [44–46]. Overall, 2,452 subjects underwent *F*eNO measurements. Two studies were performed in Europe and one in the USA. Only one study was multicentric [46]. Ethical Committee approval and informed consent were mandatory in all studies. For *F*eNO, as for BAL and induced sputum, inclusion and exclusion criteria differed largely, as one study had been conducted on unselected random subjects from the general population [44]. All studies detected *F*eNO at a flow rate of 50 $mL \cdot s^{-1}$, while the studies employed three different NO analysers (table 5).

The data from OLIN *et al.* [44], involving a large population of randomly selected adult subjects, indicated a median *F*eNO value of 16 ppb, although with a wide range (2.4–199 ppb). The authors observed an association of *F*eNO values with height and age but not with sex. However, this study also included subjects with asthma, atopy and receiving steroid treatment. In contrast, OLIVIERI *et al.* [46] specifically designed a study to address *F*eNO normal values. They measured *F*eNO in a population of 204 healthy nonatopic, nonsmoking subjects aged 19–65 yrs at a flow rate of 50 $mL \cdot s^{-1}$ using the online single breath technique and identified a mean value of 10.8$\pm$4.7 ppb. They found a sex-related difference, as values of *F*eNO were significantly lower in females at the studied expiratory flows [46]. The effect of age was also investigated by HAIGHT *et al.* [45] in a study evaluating a much smaller population; they observed increased values in older subjects (table 5).

On the basis of these data it can be stated that in healthy nonsmoking adult individuals *F*eNO levels fall within the 4–20 ppb range, and the value of 20.2 ppb (mean+2SD) can be identified as a cut-off point between normal and abnormal *F*eNO.

Problems open for discussion

Scarcity of normal value studies

Larger studies specifically addressing the problem of reference values in different normal populations (considering age, sex, smoking history, atopy, *etc.*) are still needed.

Outlier values

Some normal individuals have high *F*eNO levels: once an underlying disease is excluded, further studies are needed to clarify the reason(s) for these unexpected values [76].

*F*eNO predicted values

When a clearer picture of how important each single factor is in determining the final *F*eNO values becomes available, it might be possible to calculate a ''predicted'' normal *F*eNO level for each subject; taking into account age, sex, smoking history, *etc.*, in a similar way as for lung physiological parameters.

EXHALED BREATH CONDENSATE

The first study on EBC was published in 1980, when authors quantified indices of lipid peroxidation in EBC [24]. The initial enthusiasm for this technique was later tempered by technical and analytical difficulties, related to the low concentration of putative biomarkers detectable in EBC, the site of EBC particle formation and finally the need for dilution and salivary markers for a proper interpretation of EBC studies (table 1) [6, 20].

EBC does not contain cells but mediators and chemical compounds, which can be determined by radioimmunoassay and enzyme immunoassay. However, most of the substances detectable in EBC are present at trace level, thus requiring highly sensitive assays for detection. On this basis, particular caution should be exercised when using nonvalidated biochemical techniques to make measurements close to the limit of detectability. To improve the specificity of the measurements, additional methods such as chromatography and mass spectrometry have been employed. These sophisticated and costly techniques increase the ability to detect and quantify the many different EBC components, but reduce the future prospects of a clinical application of EBC analysis, if not carried out in specialised centres.

Recently, International Guidelines have been published with recommendations to optimise the method and achieve better standardisation and reproducibility of procedures [6]. EBC collection is completely noninvasive and thus should not encounter ethical difficulties in its widespread application to identify reference values.

Results of the literature search

In contrast to the previous considerations, somewhat surprisingly, there is no ''normal reference value'' study in the EBC literature, *i.e.* no study has specifically addressed the issue of obtaining reference data for the many molecular markers contained in EBC from normal subjects. Thus, it is not possible to define reference values for any EBC mediator, such as the current authors have highlighted for BAL, induced sputum and *F*eNO. At this stage, it is only possible to capitalise on studies dealing with EBC analysis in diseased conditions, although in these studies a limited number of healthy subjects matching the characteristics of the diseased group are usually used as a control group. In this context, a number of such publications have dealt with the same mediator, therefore, more data are available in healthy controls (table 6) [77–93]. The only exception is a recent study dealing not with a mediator or chemical compound but with pH values in healthy subjects [87].

Overall, given the nature of the matrix to be analysed and the related technical difficulties, it is not surprising that no indication for a clinical use of EBC analysis has been put forward. The field in which EBC analysis may soon have a recognised clinical application is in assessing exposure to chemical compounds potentially harmful to the respiratory system, *e.g.* in the screening of occupational/environmental risk or toxic effects of a given compound in exposed individuals [91]. This type of ''occupational'' application of EBC analysis to detect a biomarker of exposure/effect is rather analogous to the role of induced sputum in occupational asthma [94], representing an example of how the development of a new biological tool may capitalise on previous experience with other types of sample analysis.

Problems open for discussion

Lack of studies addressing the problem of normal reference values for mediators contained in EBC

This is the main problem, although historically it is due to the development of the methodology, the previously mentioned technical problems and the fact that International Guidelines were only published in 2005. Nevertheless, it would appear that large collaborative studies addressing this issue are needed.

Different components of EBC

EBC contains both volatile and nonvolatile nongaseous substances. Volatile or semi-volatile substances (*e.g.* hydrogen peroxide) have appreciable vapour pressure at body temperature and, therefore, can be easily exhaled like gases. Volatile substances in gas phase dissolve in condensed water in the EBC apparatus throughout the collection period, increasing in proportion to their delivery to the EBC apparatus. Therefore, it has been proposed to quantify volatile solutes in terms of the rate at which they are dissolved in the EBC apparatus rather than in terms of their absolute concentration. This aspect of the assessment of EBC also needs to be performed in normal volunteers.

Dilution factor(s)

EBC also contains nonvolatile substances, such as salt and proteins, which are mainly added to exhaled breath in small droplets. These are further diluted with exhaled water vapours. Droplet formation does not proceed at a constant rate and is not linearly related to water vapour production. Therefore, differences in dilution of exhaled droplets by water vapour in EBC may require the use of dilution indicators for accurate data interpretation. Some authors have suggested using salt concentrations (Na^+, Cl^-, K^+) and urea as normalisation factors, assuming these substances to be equally concentrated in airway lining fluid and serum of healthy and diseased subjects. Conductivity measurement on lyophilised EBC has also been proposed as a normalisation factor. This part of the assessment of EBC also needs to be carried out using normal volunteers.

TABLE 6 Selected compounds detected in exhaled breath condensate (EBC) in healthy adult subjects

Markers	Methods	Interpretation	Values in normal subjects	Refs
Hydrogen peroxide	Colorimetric or fluorimetric methods	Free radical production	0–0.5 μM	77–81
8-Isoprostane	EIA and gas chromatography/MS	Lipid peroxidation product	0–20 pg·mL^{-1}	82–85
pH	pH electrodes and indicator dyes	Acid-base status	7.8–8.1	86–89
MDA	Liquid chromatography-tandem mass spectrometry	Lipid peroxidation product	0–15 nmol·L^{-1}	90–92
TBARS	Spectrofluorimeter	Decomposition of lipid peroxidation products	0–0.05 μM	78, 93

Data presented in this table are not from studies specifically addressing the issue of normal reference values for the different parameters/substances present in EBC, as there are no such studies in the literature. Instead the data are from control subjects whose values were employed as comparison data with diseased subjects; the only exception is [87]. MDA: malondialdehyde; TBARS: thiobarbituric acid reactive substances; EIA: enzyme immunoassay; MS: mass spectrometry.

Potential confounding factors

A factor which should be taken into account in EBC analysis is the lack of standardised measurements which may lead to different results in separate experiments. Efforts are needed to improve the sensitivity and the specificity of putative measurements through a comparison with more valid techniques, such as those based on mass spectrometry [95, 96].

DISCUSSION AND CONCLUSIONS

The data reviewed for the four methodologies highlights a series of common features. The major one among them is their ability to provide a biomarker which can have an additional clinical value. Although the time elapsed from the first description and the number of researchers involved for each methodology are also important factors, somewhat surprisingly studies designed to obtain reference data are more numerous in the literature for older and more invasive methodologies than for the newer, less invasive ones. This fact seems to call for new and larger studies in this field.

Another possible way to increase present knowledge about the type and characteristics of the signal gained from the new, noninvasive methodologies may be to compare them with the old ones in given populations of subjects. Such comparison studies could be useful in defining the site of sampling for the different methodologies (*e.g.* lower *versus* upper airways). In addition they could be useful for correlating the presence of biomarkers obtained through invasive/expensive/time-consuming methods with new ones hopefully obtained by new, noninvasive/cheap/online methods. An example could be the inclusion in the future of normal value *F*eNO studies of the evaluation of induced sputum cells, to identify the presence, type and severity of airway inflammation and to correlate this information with that of the noninvasive biomarker. Such comparison studies have been performed for instance between induced sputum and EBC in chronic airway inflammatory diseases and between BAL and EBC in ILD [90, 97, 98], as well as between BAL and/or biopsy and induced sputum, but clearly a deeper evaluation in this field is needed before a reliable biomarker for clinical use can be identified [99].

In any case, before reaching the goal of clinical application for each methodology, many obstacles have to be overcome. The assessment of reliable normal reference values is certainly one of the most important obstacles. In this context, the data reviewed have revealed many common problems.

How to define a normal control subject?

This is not, as it might seem, an obvious or useless question since there is no agreement in the literature on the inclusion criteria for subjects enrolled as ''normal control'', even in studies designed to obtain reference data (tables 2–6). As an example, very few studies that specifically focused on normal subjects managed to ascertain a status of atopy. In western countries, atopy seems increasingly present in the general population and its presence is relevant for all the biological methodologies. Another example is the time that should elapse from an acute airways infection in normal subjects to be sure that all inflammatory parameters return to baseline values. In this context, there is evidence that induced sputum, *F*eNO and EBC may show important changes during or after a viral infection of the larger airways or a common cold [64, 100, 101]. As these changes may last for weeks, upper respiratory tract infection may act as a confounder in studies focused on reference biomarkers and, consequently, the recruited subjects should be free of such episodes for $\geqslant$1 month.

How to recruit normal volunteers?

To the current authors' knowledge there are no standard normatives in European Union countries pertaining to enrolment (and possible remuneration) of normal volunteers; although it is permitted, at least in some countries, to ''reimburse'' patients enrolled in clinical trials. Conversely, informed consent and approval from Ethical Committees are mandatory in Europe. This combined situation may cause increased difficulties in recruiting normal volunteers. As reimbursed volunteers may represent a biased population of normal subjects, *e.g.* for their social status, to what extent may the data obtained from this population be used as reference values for the general population?

Monocentre versus multicentres

The vast majority of studies designed to obtain normal reference data for each methodology are monocentric. Such studies often enrol small numbers of subjects. This may be useful since each institution should obtain its own reference values to compare this data with that obtained from the local

patient population(s). However, a mono-institutional study often suffers from the problem of insufficient population size. In many cases, large multicentre studies may be useful to compare data and to pool them in larger population samples that take into account many variables, such as sex, race, age, smoking history, *etc*.

Ethical problems

Are we permitted to use data obtained from ''control patient'' populations submitted to a given methodology as reference values in our clinical setting? To what extent may we compare studies performed employing reimbursed volunteers with studies performed where reimbursed volunteers are not allowed? Should the process of reviewing manuscripts take into account these differences?

Conclusion

In conclusion, for each methodology, and particularly for the newer ones, there may still be the need for multicentre collaborative studies designed to obtain control data from a larger population of normal subjects. As a prerequisite, the standardisation of the methodology, a well designed set of inclusion criteria and a better definition of the characteristics of the studied subjects should be agreed on, with the aim of knowing exactly how and from which populations the data to be used as reference values is obtained. In Europe, common legislation or guidelines on this issue would be very important to place all investigators under the same conditions when designing their studies. Finally, the scientific community should lobby to underscore the importance of such studies involving normal subjects. These studies are designed to increase current knowledge about basic processes ongoing in the respiratory system as a means to providing, ultimately, better care for patients.

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