Year : 2022

Novel analytical methods for the non-invasive measurement of oxidative stress in exhaled air: application to workers in underground railway enclosures

Hemmendinger Maud

Hemmendinger Maud, 2022, Novel analytical methods for the non-invasive measurement of oxidative stress in exhaled air: application to workers in underground railway enclosures

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_94B14BA72F409

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département Santé, travail et environnement, centre universitaire de médecine générale et santé publique (Unisanté)

Novel analytical methods for the non-invasive measurement of oxidative stress in exhaled air: application to workers in underground railway enclosures

UNIL PhD in Life Sciences:

Thesis report

By

Maud Hemmendinger

Jury

Dr. Fabio Candotti, President

Prof. Irina Guseva Canu, Thesis director

Dr. Guillaume Suárez, Thesis co-director

Prof. Laurent Decosterd, Expert

Dr. César Fernández-Sánchez, Expert

Dr. Michael F. Koller, Expert

Lausanne, 2022

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Ecole Doctorale
Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président·e	Monsieur	Prof.	Fabio	**Candotti**
Directeur·trice de thèse	Madame	Prof.	Irina	**Guseva Canu**
Co-directeur·trice	Monsieur	Dr	Guillaume	**Suárez**
Expert·e·s	Monsieur	Prof.	Laurent	**Décosterd**
	Monsieur	Dr	César	**Fernández-Sánchez**
	Monsieur	Dr	Michael F.	**Koller**

le Conseil de Faculté autorise l'impression de la thèse de

Maud Irène Hemmendinger

Diplôme de formation approfondie en sciences pharmaceutiques (Ph045-231), Université de Strasbourg, France

intitulée

Novel analytical methods for the non-invasive measurement of oxidative stress in exhaled air: application to workers in underground railway enclosures

Lausanne, le 20 juin 2022

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Fabio Candotti

REMERCIEMENTS

Je tiens à remercier en tout premier lieu le Président de thèse, Monsieur Fabio CANDOTTI, qui me fait l'honneur d'accepter la présidence de ce jury. Mes sincères remerciements vont également à Monsieur Laurent DECOSTERD, Monsieur Michael KOLLER et Monsieur César FERNANDEZ qui me font l'honneur en acceptant de juger ce travail de thèse.

Mes remerciements vont également à mes deux directeurs de thèse Madame Irina GUSEVA CANU and Monsieur Guillaume SUAREZ, pour m'avoir accueillie, guidée, et soutenue dans ce travail. J'ai beaucoup évolué durant ces quatre années à vos côtés, gagné en maturité et acquis une forte rigueur scientifique. Merci encore pour tous vos conseils avisés, votre extrême patience et vos encouragements dans l'écriture de cette thèse. Vous avez été un réel moteur, en mettant à ma disposition tous les moyens nécessaires pour valoriser ce travail sous forme de nombreuses publications. C'était un honneur d'avoir pu travailler à vos côtés.

Je tiens à remercier toute l'équipe de la RATP, pour leur collaboration et sans qui tout ce travail aurait été impossible. Un merci particulier au médecin Dr. Amélie DEBATISSE pour la coordination des campagnes de prélèvement sur le terrain et pour l'aide qu'elle m'a apportée. Merci également aux infirmiers et aux équipes techniques, qui m'ont accompagnée avec un grand professionnalisme, et qui m'ont apporté leur savoir-faire.

Mes plus vifs remerciements vont aussi aux autres personnes qui, de près ou de loin, ont contribué à ce travail, en particulier Monsieur Jean-Jacques SAUVAIN. Merci d'avoir toujours gardé ta porte ouverte face à mes (nombreuses) sollicitations, relatives aux questions analytiques, ou même parfois à quelques préoccupations personnelles. Merci encore pour avoir partagé ton temps précieux et ton immense savoir. Un grand merci à tous mes collègues du Bugnon 19 : Unisanté et laboratoire. En particulier Nicolas S., Nicole, Antonio, Simon, Nicolas C. L., Gregory qui ont contribué à la bonne ambiance générale. Je n'oublierai également jamais les discussions philosophiques que nous avons eus à la cafeteria et que je ne manquais pour rien au monde. Petite mention à Nicolas S., pour sa bonne humeur et avec qui j'ai partagé de nombreux fous rires dans le bureau (qui parfois s'entendaient dans tout le bâtiment...).

Je remercie enfin les nombreuses autres personnes sans lesquelles ce travail n'aurait pu être achevé en m'apportant un soutien indéfectible et qui ont contribué à façonner la personne que je suis aujourd'hui. Un grand grand merci à ma famille, mes deux grands-mères Carmen et Margot, mes parents, Hugues et Carine et mon frère Arnaud. Tous mes amis Céline, Nicole, Manon, Jason… et plus particulièrement mon compagnon Raphaël, qui est toujours resté présent pour moi, même dans les moments les plus difficiles.

TABLE OF CONTENTS

REMERCIEMENTS i
LIST OF FIGURES v
LIST OF TABLES x
ABBREVIATIONS xii
ABSTRACT xiv
RÉSUMÉ xv
THESIS OVERVIEW xvi
BACKGROUND AND RATIONALE 1
I. Chronic Obstructive Pulmonary Disease (COPD) 1
1. Description of the disease 1
2. Factors that influence chronic obstructive pulmonary disease development and progression 3
3. Current diagnosis of chronic obstructive pulmonary disease 10
4. Limitations in the current diagnostic approach 13
II. Emerging biomarkers in chronic obstructive pulmonary disease diagnosis 14
1. Definition and typology of biomarkers 14
2. Constraints in the implementation of new biomarkers 17
3. Oxidative stress in chronic obstructive pulmonary disease 19
III. Oxidative stress 19
1. Pathophysiology of oxidative stress 19
2. Reactive oxygen species detection 21
IV. Oxidative stress biomarkers 22
1. Lipoperoxidation 22
2. Nucleic acid oxidation 27
V. Matrices for biomarker analysis 28
1. Urine 29
2. Exhaled breath condensate 29
3. Oxidative potential in exhaled air 37
FRAMEWORK OF THE THESIS 38
STANDARDIZATION OF BIOMARKERS DETECTION TECHNIQUES IN EXHALED AIR 42
I. Improvement of existing analytical methods in exhaled breath condensate 42
1. Development of method for MDA 43
2. Development of method for 8-OHdG/8-isoprostane 62
II. Optimize and characterize the determination of oxidative potential in the exhaled air - OPEA 78

1. Context....78
2. Methods....78
3. Experimental optimization of the OPEA measurement....84
FEASIBILITY OF SELECTED OXIDATIVE STRESS BIOMARKERS IN A PILOT FIELD....93
I. Context....93
1. Contribution to this part....93
2. Aims and design of the studies....93
II. Materials and methods....94
1. Research setting and participants....94
2. Exposure and health outcome measures....96
3. Description of exposure devices....104
4. Statistical analysis....112
III. Pilot study results....115
1. My contribution to the pilot study....115
2. Valorisation of the pilot study results....115
3. Summary of the main results from pilot study....116
4. My contribution to the Field study....134
REFERENCE VALUES FOUND IN THE LITERATURE....135
I. Context....135
II. Overview of the 8-OHdG results....137
1. Methods....138
2. Results....142
III. Overview results for 8-isoprostane....151
1. Context....151
2. Results....151
GENERAL DISCUSSION....163
I. Methodological aspects....163
1. 8-OHdG/8-isoprotane method optimization....163
2. MDA method optimization....163
3. OPEA optimization....165
II. Experience gained from the method application on the pilot field study....166
1. 8-OHdG/8-isoprostane....166
2. MDA....169
3. OPEA....171
4. Strengths of the pilot study....172
5. Limitations of the pilot study....173
PERSPECTIVES AND FUTURE WORKS....174

CONCLUSION 175
VALORIZATION AND COMMUNICATION OF THE RESULTS 176
REFERENCES 180
ANNEXE 206

LIST OF FIGURES

Figure 1. *Histopathological images of rat lung tissue slices from [10] captured under a microscope. Magnification, ×200. (a) Control, (b) COPD. While, the bronchial lumen and alveolar structure are normal in control, in COPD bronchial lumen is distorted and damaged, characterized by goblet cell hyperplasia, inflammatory cell infiltration. The alveolar septum is thickened due to a compensatory emphysema*..... 2
Figure 2. *High-resolution computed tomography scans from [11] of (A) control, (B) COPD with an evenly distributed pulmonary emphysema*..... 2
Figure 3. *Different aerodynamic diameters according to their origin [44]*..... 5
Figure 4. *Different aerosols with different sizes and shapes* 7
Figure 5. *Agglomerated particle of Diesel [68]* 8
Figure 6. *Illustration of particles-induced oxidative stress and inflammation in pneumocytes and macrophages*..... 9
Figure 7. *Spirometry testing for differentiating obstructive from normal pattern. Illustration modified from [91]*.....11
Figure 8. *Biomarkers of exposure, effect and susceptibility modified from* [111] 15
Figure 9. *The number of biomarkers from discovery to clinical use*.....18
Figure 10. *The hierarchical oxidative stress model from [131]* 21
Figure 11. *IsoPs formation from* [147].....24
Figure 12. *Formation of malondialdehyde [158]*.....25
Figure 13. *Reaction of 2'-deoxyguanosine with hydroxyl radicals [173]* 28
Figure 14. *Collection of EBC*.....31
Figure 15. *Collection of respiratory droplets through condenser [185]. Variable quantities of droplets are released from the surfaces of the airways in the form of water vapor according to the polarity. In the second picture, the respiratory droplets reach the condenser, they deposit on its cold walls.* 33
Figure 16. *MDA derivatization process with DNPH yielding 1-(2,4-dinitrophenyl) pyrazole (MDA-DNPH). At lower pH MDA appears in equilibrium between its protonated enol (α-β-unsaturated carbonyl) aldehyde form and the dialdehyde form*.....43
Figure 17. *Validation sequence in three different days*.....46
Figure 18. *Effect of the reaction temperature on the MDA-DNPH formation as a function of time. The small box represents the variation of the MDA concentration at 20°C over a period of 24 hours* 47
Figure 19. *Contribution of the acid type on the signal of MDA-DNPH in procedural blanks. The error bars correspond to the standard deviation of five measurements. Comparisons were*

*made by one-way ANOVA followed by Tukey's test (*p< 0.05; **p< 0.005). AcOH: acetic acid; ACN: acetonitrile.* 52
Figure 20. *Effects of the added DNPH concentration expressed in mM on a) the peak area of MDA-DNPH b) the peak area of MDA-d2-DNPH for a standard solution at 35 ng/mL. The error bars correspond to the standard deviation of three measurements.*.....53
Figure 21. *Effect of MDA-d2 concentration on the peak area of MDA-DNPH. For a standard solution at 0 ng/ml. The error bars correspond to the standard deviation of four measurements* 54
Figure 22. *a) Chromatogram of procedural blank and its internal standard and multi-reaction monitoring (MRM) transitions for MDA b) Partial chromatogram of a procedural blank, of the lowest standard MDA concentration and of a typical EBC sample, with retention times (RT). The units of the y-axis are in arbitrary units, counts per second.* 55
Figure 23. *Accuracy profile for MDA analysis in EBC represented in the form of box-and-whisker plot. The figure denotes the measured relative error compared to the targeted spiked concentration. The black central line represents the median value (50th percentile) whereas the red dashed lines represent the average value. The upper and lower whiskers correspond to the adjacent values as defined by Tukey [234]. Data points falling outside the adjacent values can be considered as potential outliers and are plotted separately. The external dashed lines correspond to the acceptance limits (fixed at 20%).* 58
Figure 24. *The relative standard uncertainty contribution for each parameter considered in the uncertainty measurement* 59
Figure 25. *The calculated expanded uncertainty, using a coverage factor of 2, as a function of the MDA concentration in EBC* 60
Figure 26. *Stacked bar graph representing the percentage of values per participant below the LOD (light blue), between the LOD and LOQ (blue) and above the LOQ (dark blue)* 61
Figure 27. (a, b) *Partial chromatogram of a standard injection at 5pg/mL (left), of the lowest quality check (spiked EBC at 5 pg/mL, middle) and of a typical EBC sample (right), with retention times (RT) for 8-isoprostane and 8-OHdG, respectively. The units of y-axis are in arbitrary units, counts per second. (a, b) are chromatograms obtained from the same healthy volunteer.* 71
Figure 28. *Slope signal (not corrected with IS) of the calibrations (minimum of 5 levels) obtained with water and EBC after lyophilization (lyoph) or vacuum concentration, in comparison with standard solutions without any concentration step for the biomarker a) 8-OHdG and b) 8-isoprostane, respectively. Error bars correspond to a minimum of two independent repetitions. Au= Arbitrary unit; Lyoph = lyophilization; standard (= non-concentrated standard); H2O lyoph (=concentrated standard).* Statistically significant difference (p< 0.05) compared to the standard without the concentration.* 72

Figure 29. *Effect of using the centrifugal concentrator system on the signal of both analytes in EBC. Bars correspond to standard deviation of three independent repetitions. CCS= centrifugal concentrator system* 73
Figure 30. *Effect of Tween-20 treatment on material surface on the signal (not corrected with IS) of 8-OHdg and 8- isoprostane. Error bars correspond to a minimum of nine independent repetitions.*.....74
Figure 31. *Efficiency of multi-scattering amplification of absorbance strategy compared to standard colorimetric assays based on Beer-Lambert Law on analytical performances of the FOX assay at low H_2O_2 range a) calibration curves based on multi-scattering amplification and internal-reflection b) calibration curves based on Beer-Lambert absorption performed with UV-Spectrophotometer.*.....79
Figure 32. *Schematic representation of the first-generation photonic system - without instrumentation - for measuring the oxidizing potential on an external amplification mode in which a Teflon diffusing insert inserted in an aluminum-reflecting cavity allows an increase in sensitivity and the analysis of the total volume of the reaction medium.* 81
Figure 33. *Exhaled air sampling system comprising the disposable mouthpiece (1) connected to a capnometer valve (2) which allows the alveolar fraction of the sample to be recovered in a 1L Tedlar bag* 82
Figure 34. *Descriptive picture of OPEA analyzer controlled with Arduino software. The air sample bubbling and the OPEA analysis occurs in two consecutive steps. EA sample from the volunteer is directly collected in the TEDLAR bag (bottom right) and plugged to the OPEA system for analysis. The air sample is forced to bubble in D_2O (1 mL) during 4 min (250 mL/min). Then, the analysis is performed by introducing 300 µL of the collection solution into the reaction vial containing 700 µL of FOX prior to the photonic measurements (3 min). Triplicate measurements can be made per air sample.*.....83
Figure 35. *Calibration curve obtained with OPEA analyzer with [H_2O_2] ranging from 0 to 1 µmol in 1 mL of FOX reactive reagent. For readability, only the curve fraction from 0 to 10 nmol is shown in logarithmic scale. For each [H_2O_2] triplicates were used and the corresponding error bars are shown on the curve* 84
Figure 36. *Contribution of the water type on the slope in blanks samples. The error bars correspond to the standard deviation for a minimum of three measurements* 86
Figure 37. *Reduction of optical interferences by the use of a near-infrared (750 nm) light source as normalizing signal* 87
Figure 38. *Descriptive picture of OPEA analyzer with three measurement channels controlled with Arduino software utilized during field campaign*.....88
Figure 39. *Example of Splunk a) graphical reports b) OPEA dashboard. Possibility to select the day and the measurement replicate (1 to 3), indication of the number of individuals*

measured during the day for each measure (denoted by an id) is associated a slope, an ordinate and a regression line. 89
Figure 40. *Schematic representation of the procedure followed by sub-study indicating the time points for EBC and EA collection on two groups of participants: healthy (n=5) and COPD (n=5) volunteers* 90
Figure 41. *Box-plot representation of overall OPEA values (n=18; n=19) obtained for the four volunteers with low OPEA values as expected for healthy controls (values corresponding to volunteer_2 are excluded). The ambient OP values correspond to indoor environments (laboratory) measured at the different time-points of the study. OPEA_AA = alveolar fraction of OPEA, OPEA_AT= total fraction of OPEA.* 92
Figure 42. *Preparatory actions and measurements carried out every day for each participant during two-week prospective follow-up in the pilot study*.....97
Figure 43. *Biological equipment at RATP sampling place for pilot study* 98
Figure 44. *Example of form for urine sampling given to the participant* 100
Figure 45. *Results of EA measurements classified according to the date, weekday, the raw file, id, regression line, the slope as well as the ordinate and* R^2 101
Figure 46. *Two RATP technician equipped with a) personal samplers for gravimetry (PM2,5; PM10; Cr III), oxidative potential (particle and volatile fractions) and monocyclic aromatic hydrocarbons (MAH) as well as direct reading instruments for relative humidity and temperature (EcoLog). b) Ultrafine particles (DiSCmini), OC (*PM_4*), GRIMM, individual dust collector Cip10 and SEM* 103
Figure 47. *Direct-reading equipment at Châtelet place* 104
Figure 48. *Micronuclei frequency from cell in buccal mucosa sampling* 110
Figure 49. *Associations to be considered during statistical analysis of the field study data, numbered by order of investigation. The dependent variables corresponding to the studied biological or health effects are shown in bold* 113
Figure 50. *Effect of the professional activity on the predicted EBC levels of MDA* 126
Figure 51. *Predicted values for alveolar OPEA (pmol/L)* 133
Figure 52. *Ambient corrected alveolar OPEA (pmol/L)*.....133
Figure 53. *Ba concentration in PM10 as a function of total OPEA during the day (pmol/L)* 134
Figure 54. *Flow-chart of study selection*.....143
Figure 55. *Forest-plot of the 8-OHdG levels [pg/mL] measured in the exhaled breath condensate according to the analytical method* 149
Figure 56. *Forest-plot of the 8-OHdG levels (pg/mL) measured in the exhaled breath condensate according to the smoking status and analytical method*.....150
Figure 57. *Flow chart of study selection*.....152

Figure 58. *Forest-plot of the 8-isoprostane levels [pg/mL] measured in the exhaled breath condensate using immunological analytical methods and in studied with only females or only males (n=13)* 159
Figure 59. *Forest-plot of the 8-isoprostane levels [pg/mL] measured in the exhaled breath condensate using immunological analytical methods and in studies with both males and females (n=39)* 160

LIST OF TABLES

Table 1. *Classification of airflow limitation severity in COPD in patients with $FEV_1/FVC < 70\%$* 12
Table 2. *ABCD system for assessment of symptoms/risk of exacerbations*.....13
Table 3. *Non-exhaustive list of reactive oxygen species* 20
Table 4. *Non-exhaustive list of analytes present in the EBC* 30
Table 5. *Recommendations from American Thoracic Society (ATS)/European Respiratory Society (ERS) for collection and EBC analysis* 34
Table 6. *Reported concentrations of 8-isoprostane in EBC of COPD patients versus controls* 36
Table 7. *Reported concentrations of 8-OHdG in EBC of COPD patients versus controls* 36
Table 8. *Reported concentrations of MDA in EBC in of COPD patients versus controls*.....37
Table 9. *Quantification of the uncertainty components at LOQ (209 pg/ml)*.....51
Table 10. *Validation parameters determined for the analysis of MDA in EBC following the FDA/ICH guidelines*.....57
Table 11. *Concentrations of MDA in 164 EBC samples from nine healthy individuals from three different professional categories, collected over two months* 60
Table 12. *Description of the standard preparation with the corresponding concentrations* ...64
Table 13. *MS transitions and instrumental conditions for 8-OHdG and 8-isoprostane analysis. The vaporizer temperature was 350°C with the ion transfer tube set at 390°C. The argon gas pressure was set at 1.5 mTorr* 66
Table 14. *Summary of participant demographics (n=26)* 68
Table 15. *Main figures of merit of the analytical method for 8-OHdG in EBC* 75
Table 16. *Main figures of merit of the analytical method for 8-isoprostane in EBC* 76
Table 17. *Results obtained for the calibration, QC and EBC samples* 77
Table 18. *OPEA (alveolar (OPEA_AA) and total air fractions (OPEA_AT) and ambient OP values calculated for healthy controls (n=5) at different sampling time and under different environments, as described in the protocol of Figure 39. All OPEA and OP ambient values are expressed in pmol/L_{air}*.....91
Table 19. *Simulation of the number of participants*.....96
Table 20. *Indoor air exposure measurements performed in the longitudinal pilot study in a Parisian subway* 106
Table 21. *Biomarker measures performed in the longitudinal pilot study in the Parisian subway* 108
Table 22. *The course of the typical medical visit in the field study*.....111

Table 23. *Primary and secondary health outcome definition and measurement in the field study in the Parisian subway*.....114
Table 24. *Estimated geometric concentration (with 95% confidence interval values into bracket) of the different fractions (ultrafine, $PM_{2.5}$, respirable and inhalable dusts) measured for the three different professional groups, as well as the UFP: Ultrafine particle number, size and lung deposited surface area. Concentration of different elements (aluminium, iron, copper, manganese, arsenic, cadmium, chrome, lead, nickel, barium and zinc) in the PM_{10} and $PM_{2.5}$ fraction. Concentration of organic and elemental carbon in respirable fraction* 118
Table 25. *Proportion of different elements (aluminum, iron, copper, manganese and zinc) in the PM_{10} and $PM_{2.5}$ fraction*.....121
Table 26. *Estimated geometric concentration or average concentration ± 95% confidence interval into bracket of the selected variables in EBC samples collected during the two working weeks for the three professional categories. The concentrations are compared either to regulatory values when they exist, or to guide values in the literature*.....122
Table 27. *Estimated geometric concentration or average concentration ± 95% confidence interval into bracket of the selected variables in urine samples collected during the two working weeks for the three professional categories. The concentrations are compared either to regulatory values when they exist, or to guide values in the literature.*.....127
Table 28. *Correlation between EBC levels of MDA and the different anions* 131
Table 29. *Association between personal exposures and MDA at lag 0 or 24 hours after exposure (lag 1)* 132
Table 30. *Quality appraisal and scoring criteria*.....140
Table 31. *Summary of studies included into systematic review - meta-analysis* 145
Table 32. *Description of the included high quality studies* 153
Table 33. *Mixed effect regression analysis to investigate the relation between levels of 8-isoprostane in EBC [pg/mL] and device used during collection, gender, BMI, smoking status, and mean age of the population* 161
Table 34. *Mixed effect regression analysis to investigate the relation between levels of 8-isoprostane in EBC [pg/mL] and device used during collection, BMI, smoking status, and mean age of the population*.....162
Table 35. *8-isoprostane reference ranges [pg/mL] in the exhaled breath condensate (EBC) of healthy adults* 162
Table 36. *Value of LOD resulting of three common methods of calculation*.....165
Table 37. *Descriptions of the analytical methods previously developed to analyze MDA in EBC with DNPH derivatization*.....169

ABBREVIATIONS

8-OHdG	Eight-hydroxy-2-deoxyguanosine
AA	arachidonic acid
ATS	American Thoracic Society
BAL	bronchoalveolar lining fluid
CAST	Cardiac Arrhythmia Suppression
COPD	chronic obstructive pulmonary disease
DNA	deoxyribonucleic acid
EA	exhaled air
EBC	exhaled air condensate
EFS	underground railway enclosures
ELISA	enzyme-linked immunosorbent assay
ERS	European Respiratory Society
H_2O_2	hydrogen peroxide
ICRP	International Commission on Radiological Protection
IsoPs	isoprostanes
LDL	low density lipoproteins
LDSA	lung deposited surface area
LOD	limit of detection
LOQ	limit of quantification
MAPK	mitogen-activated protein kinase
MDA	malondialdehyde
Nrf-2	nuclear factor erythroid 2 related factor 2
OEL	occupational exposure limit
OPEA	oxidative potential exhaled air
OS	oxidative stress
PAH	polycyclic aromatic hydrocarbons
PM	particulate matter
PNOS	particulate not otherwise regulated
PSA	prostate specific antigen

PUFA	polyunsaturated fatty acids
RATP	Autonomous Parisian Transportation Administration
ROBOCoP	Respiratory disease occupational biomonitoring collaborative project
ROS	reactive oxygen species
UFP	ultrafine particles
WHO	World Health Organization

ABSTRACT

Inhalation of particulate matter, via an immune mechanism, tends to induce inflammation and endogenous ROS production. ROS overproduction, in turn, lead to oxidative stress, inflammation and ultimately increase the risk of COPD. COPD is assessed and monitored by breathing tests such as spirometry. These tests often reveal the consequences of the disease at a late stage.

Medical surveillance of employees exposed to high doses of pollutants and therefore with an increased risk of developing lung diseases is justified in particular for the purpose of prevention and as early as possible in order to implement the necessary measures to avoid the pathology or its aggravation. Similarly, for COPD patients, monitoring could prevent the occurrence of exacerbations and help the clinician in the treatment.

In this context, direct access to the lung by assessing biomarkers of oxidative stress in exhaled air could be of interest. Methods that focus on the oxidative potential or on specific oxidative stress biomarkers in exhaled breath condensate (EBC) such as MDA, 8-OHdG, 8-isoprostane, are developed during this thesis. They are the subject of increasing scientific interest in recent years, as they are non-invasive and thus easily applicable in the screening of the population.

The thesis objectives were to standardize these methods via a thorough validation and to validate them via feasibility tests as a first step in an epidemiological pilot study in comparison with a large panel of exposure metrics. In this respect, our laboratory partnered with RATP (Autonomous Parisian Transportation Administration) and created a common project: the Respiratory disease Occupational Biomonitoring Collaborative Project (ROBoCoP). The RATP's underground rail enclosures are identified as a particularly particulate polluted area. Exposures to such high concentrations of particulate matter over a prolonged period of time may significantly increase the risk for RATP workers of developing COPD.
We were able to detect MDA in all RATP EBC samples at very low concentration close to LOQ. (211 pg/ml) and was associated with PM_{10} metric. Concerning 8-OHdG/ 8-isoprostane the results were under LOD (< 1 pg/ml). We suspected that the lack of detecting these biomarkers in our study was due to factors affecting EBC collections. Concerning the OPEA metric we observed no correlation with any of the measured exposure variables.
In conclusion, MDA seems to be a relevant biomarker of exposure contrary to OPEA and 8-OHdG/ 8-isoprostane. Nevertheless, we believe that OPEA would be particularly suitable – as stated by earlier pilot study– to predict inflammatory exacerbation episodes or early diagnosis of COPD. Future studies might be conducted in order to address such hypotheses and determine for these biomarkers the pertinent field of use.

RÉSUMÉ

L'inhalation de particules provoque, via un mécanisme immunitaire, la production de ERO (espèces réactives de l'oxygène) endogènes. La surproduction de ERO peut conduire à un stress oxydant, à de l'inflammation et finalement augmenter le risque de développer des maladies pulmonaires telle que la bronchopneumopathie chronique obstructive (BPCO). L'évaluation et le suivi de la BPCO repose actuellement sur un test d'exploration fonctionnelle respiratoire (spirométrie). Malheureusement, ce test met souvent en évidence, parfois tardivement, les conséquences de la maladie.

La surveillance médicale de salariés exposés à des concentrations importantes en particules est importante car ils ont un risque accru de développer une BPCO. Cette surveillance doit être le plus précoce possible pour permettre de mettre en œuvre tous les mesures nécessaires pour éviter la survenue de la pathologie ou même son aggravation. En ce qui concerne les patients déjà atteints de BPCO, la surveillance peut permettre de prévenir la survenue d'épisodes d'exacerbation et aider le clinicien dans le traitement qu'il pourrait proposer.

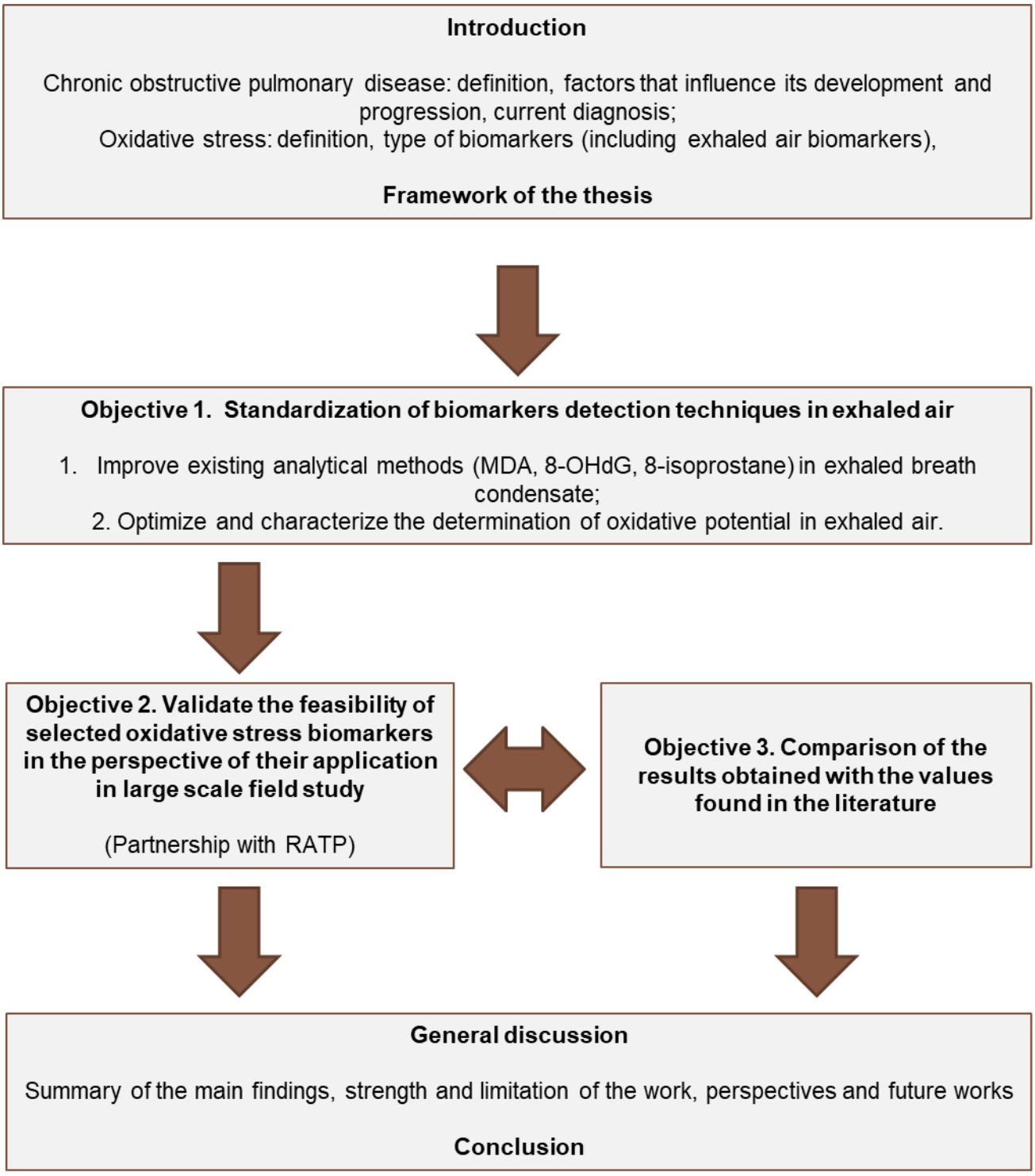
Dans ce contexte, un accès direct au poumon par l'évaluation de biomarqueurs du stress oxydant dans l'air exhalé pourrait être intéressant. Ces biomarqueurs font l'objet d'un intérêt scientifique croissant ces dernières années, car ils sont non-invasifs, facilement applicables pour tout type de patient et donc pratiques pour des études de dépistage. Ils peuvent permettre d'estimer le degré d'atteinte du poumon avant l'apparition ou l'exacerbation des effets cliniques. Des méthodes qui permettent l'évaluation du potentiel oxydant (OPEA) ou de la quantification des biomarqueurs spécifiques du stress oxydant dans le condensat d'air exhalé (EBC) tels que le MDA, le 8-OHdG et le 8-isoprostane, ont été développées au cours de cette thèse.

Les objectifs de la thèse étaient de standardiser et de valider ces méthodes via des tests de faisabilité durant une étude pilote épidémiologique. Dans cette optique, notre laboratoire s'est associé à la RATP (Régie autonome des transports parisiens) pour constituer le projet ROBoCoP (Respiratory disease Occupational Biomonitoring Collaborative Project). Les enceintes ferroviaires souterraines de la RATP sont identifiées comme une zone particulièrement polluée par les particules. L'exposition à ces particules sur une période prolongée peut augmenter de manière significative le risque de développer une BPCO.

Lors de cette étude, nous avons pu détecter le MDA dans tous les échantillons d'EBC de la RATP à de très faibles concentrations proche de la LOQ (211 pg/ml). Ces concentrations de MDA étaient corrélés avec les PM_{10}. Concernant le 8-OHdG/ 8-isoprostane, les résultats étaient inférieurs à la LOD (< 1 pg/ml). En ce sens, nous pensons que l'absence de détection de ces deux biomarqueurs pourrait être due à des facteurs affectant la collecte d'EBC. En ce qui concerne la mesure de l'OPEA, nous n'avons observé aucune corrélation avec les variables d'exposition mesurées dans les enceintes ferroviaires souterraines, ce qui pourrait compromettre son utilisation en tant que biomarqueur d'exposition.

En conclusion, le MDA semble pertinent en tant que biomarqueur d'exposition contrairement à l'OPEA et au 8-OHdG/ 8-isoprostane. Néanmoins, nous pensons que l'OPEA serait plutôt adapté pour prédire les épisodes d'exacerbation inflammatoire, pour le diagnostic précoce de la BPCO, comme suggérée lors d'une précédente étude. De futures études devront néanmoins être menées afin de confirmer ces hypothèses et de déterminer le domaine d'application de ces biomarqueurs.

THESIS OVERVIEW



BACKGROUND AND RATIONALE

I. Chronic Obstructive Pulmonary Disease (COPD)

1. Description of the disease

COPD is a major and increasing global health problem. It is the third leading cause of death worldwide [1], the second contributing disease of disability-adjusted life-years lost [2], and the most common cause of respiratory failure. More than 3 million people died of COPD in 2019 accounting for 6% of all deaths globally [1]. COPD prevalence, morbidity and mortality vary across countries. In Europe, the prevalence of COPD is estimated to be 12.38% [3] with an annual burden of 38.6 billion euros [4]. In Switzerland, COPD prevalence is particularly high, reaching 15.0% in adult male population [5]. The prevalence of COPD is twice as high in men as in women [6]. COPD represents an important public health challenge because it is a common, preventable and treatable disease.

COPD is a general term for a heterogeneous disease characterized by chronic airflow limitation that is usually progressive and persistent respiratory symptoms associated with an enhanced inflammatory process in the airways and/ or alveolar abnormalities [7]. The most common respiratory symptoms include dyspnea, cough, wheezing and change in sputum production. The airflow limitation is caused by inflammation causing structural changes resulting in parenchymal destruction (emphysema) [8], obstructive bronchiolitis [9], chronic mucus hypersecretion, and small airways thickening, mucociliary dysfunction and the destruction of alveolar walls and attachments that normally help to maintain airway patency. These conditions result in a decrease in elastic properties of the lungs and thus preventing the ability of the airways to remain open during expiration.

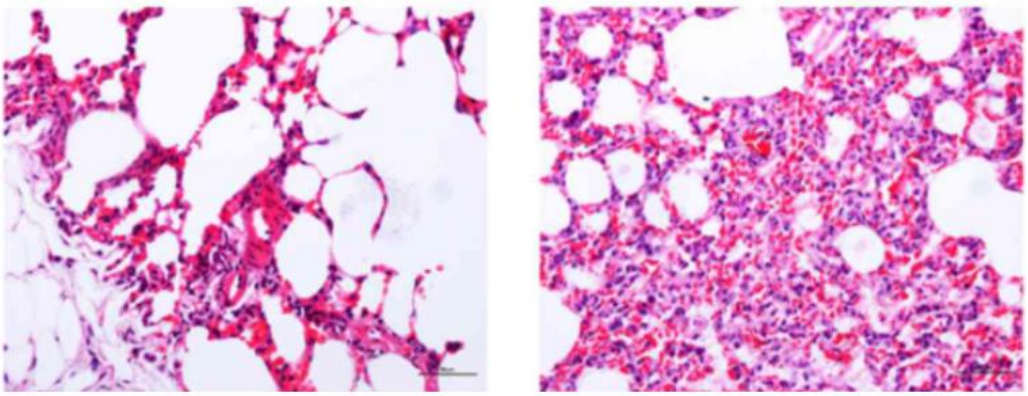


Figure 1. *Histopathological images of rat lung tissue slices from [10] captured under a microscope. Magnification, ×200. (a) Control, (b) COPD. While, the bronchial lumen and alveolar structure are normal in control, in COPD bronchial lumen is distorted and damaged, characterized by goblet cell hyperplasia, inflammatory cell infiltration. The alveolar septum is thickened due to a compensatory emphysema*

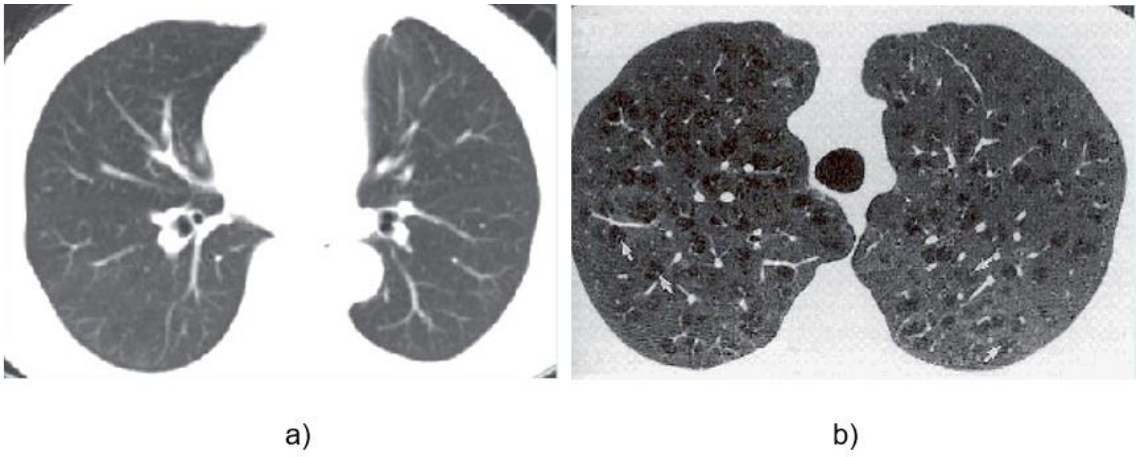


Figure 2. *High-resolution computed tomography scans from [11] of (A) control, (B) COPD with an evenly distributed pulmonary emphysema*

Patients with COPD often have important concomitant chronic illness including hypertension, cardiovascular disease [12,13], osteoporosis, pneumonia, musculoskeletal impairment, diabetes, depression and anxiety [14,15] that represent important determinants of morbidity development and mortality notably in the elderly. The existence of COPD itself may actually increase significantly the risk of mortality associated with urban air pollution due to a more substantial susceptibility to particles [16,17]. It can also increase the risk for other extra pulmonary diseases, including arrhythmia [18], metabolic abnormalities [19] and skeletal

muscle dysfunction, muscle wasting [20] and lung cancer [21]. These extra pulmonary diseases can contribute significantly to limited exercise capacity and poor health status in patients with COPD [22]. Because of their importance in the pathology of COPD, comorbidities should be routinely investigated, and appropriately treated in each patient with COPD.

The severity of COPD is also associated with disease exacerbations. Exacerbation is defined as an event in the natural course of the disease that is characterized by a change in the patient's baseline dyspnea, cough, or sputum beyond day-to-day variability [23]. Exacerbations of respiratory symptoms are caused and triggered by a variety of factors including respiratory infections with bacteria [23] or viruses [24], air pollutants, or other less studied factors. During exacerbations, there is an increased acute airway inflammation, that in turn can lead to the lung hyperinflation and air trapping, resulting in a sharp increase in the intensity of dyspnea to intolerable levels [25]. The presence of lung over-inflation triggers or worsen existing comorbidities for COPD patients because it primarily affects both cardiac and respiratory functions. The presence and frequency of exacerbations is one of the most important determinants on quality of life and activities of daily living [26]. Therefore, COPD exacerbations are an important cause of hospital admissions. Some people can cope with this disease for years while other patients are prone to frequent exacerbations, suffer and then die prematurely from acute worsening of respiratory symptoms. It is estimated that COPD patients suffer 1–4 exacerbations per year [27]. Such decompensation episodes have an important impact in emergency services, where 10% of medical admissions are attributable to acute exacerbations [28]. These hospital admissions result in a high mortality rate in COPD patients, ranging from 22–43% after 1 year to 36– 49% after 2 years, depending on the severity of the pathology [29-31].

According to the WHO estimates, the COPD burden is expected to increase over the coming decades with over six million deaths per year by 2060 [32] because of continued exposure to COPD risk factors such as increasing prevalence of smoking in developing countries.

2. Factors that influence chronic obstructive pulmonary disease development and progression

a. Individual factors

The complex interaction between individual characteristics and environmental exposures may predispose a subject to COPD. The presence of COPD at-risk populations will depend on the following individual characteristics, which attribute them a susceptibility per se to develop the disease and/or possible COPD over-worsening:

- Biological characteristics, such as age, are often cited as a risk factor for COPD, although it remains unclear whether healthy aging leads to COPD or whether age itself reflects the sum of cumulative exposures over a lifetime [33].
- Sex of the subjects has also an impact because of gender difference in immune pathways. Women have higher susceptibility to the effects of tobacco compared to men with an equivalent quantity of cigarettes consumed [34,35],
- The pre-existing pathologies (e.g., diabetes, asthma [36], coronary heart disease, obesity), and the presence of a genetic risk factor (in particular with the deficiency of alpha-1 antitrypsin, a major circulating inhibitor of serine proteases [37]);
- Specific behaviors, such as smoking or alcohol consumption; Cigarette smokers have a higher prevalence of respiratory symptoms and abnormal lung function. Smoking increases the annual rate of lung function decline, both in males and in female (23-38 ml) [38] and increases the mortality rate from COPD when compared to never-smokers The sex-adjusted relative risk of COPD is 2.89, (95% CI 2.63-3.17) for cigarette smokers compared to adults who had never smoked [39]. Exposure to passive smoking is also associated with an increased prevalence of COPD and a poorer lung function by increasing the total burden of inhaled particles and gases in the lungs [40].
- Sociological characteristics (social categories, stress). Poverty or lower socio-economic status is strongly associated with an increase of prevalence of airflow obstruction [41] and the risk of developing COPD. The mechanisms by which poverty is associated with airflow obstruction are unknown, this trend could reflect increasing exposures to indoor and outdoor air pollutants in dusty jobs or household, poor diet/lower BMI, infections (e.g., tuberculosis), higher tobacco consumption or other factors related to low socio-economic status.
- Environmental characteristics with co-exposure to other environmental factors (weather conditions, season);

b. Exposure to particles

Particle definition

Particles represents an extremely complex mixture of organic and inorganic substances in a solid, liquid or solid and liquid form suspended in a gas. The aerodynamic[1] diameter of

[1] The diameter of a particle is defined as a function of the sedimentation rate of the particles, commonly called aerodynamic diameter. This unit of length allows us to describe the dynamic behaviour of our particles of interest in a fluid such as air (aerosol) by comparing it to the diameter of a spherical particle of density equal to 1g/cm^3 having the same falling speed in a fluid at rest as our characterized particles (Strokes diameter).

particles can vary from less than 10 nm to 100 µm. Above the latter value, the particles are no longer held in suspension by air resistance. Particles with a median aerodynamic diameter <10 µm can be categorized according to their size based on a) their observed modal distribution [42]; b) the 50% cut-off diameter of the measurement instrument. In the latter case, the most common division are $PM_{0.1}$ (Ultrafine particles (UFP) and nanoparticles), $PM_{2.5}$ (fine particles) and PM_{10} (coarse particles). For example, PM_{10} is defined as airborne particulate matter passing through a sampling inlet with 50% efficiency cut-off at 10 µm aerodynamic diameter that transmits particles below this size [43]. This classification of the particulate matter size is related to its ability to penetrate to the lower part of the human respiratory tract (Figure **3**).

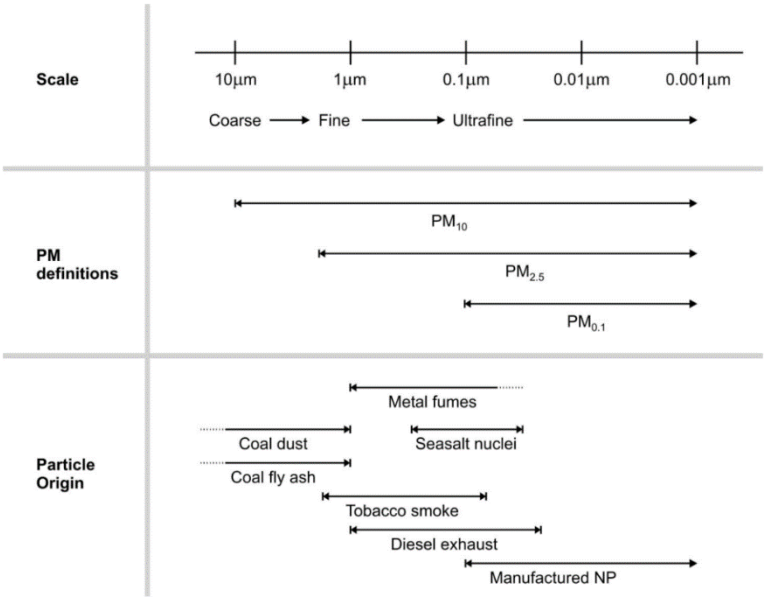


Figure 3. *Different aerodynamic diameters according to their origin [44]*

Most of the particle mass is usually found in the coarse particle fraction, while the largest number of particles is found in the ultrafine particle fraction, smaller than 100 nm. The latter contribute only a few percent of the mass, yet at the same time account for more than 90% of the number of particles. This observation can have an impact whether the toxicological effects depend on the number or the mass of inhaled particles.

Coarse particles, because of their mass, sediment relatively quickly under the effect of gravity. The ultrafine particles $PM_{0.1}$ have a fairly short life span because they will grow quite rapidly through the condensation of metallic organic vapours (condensation nuclei) or coagulate when they come into contact with pre-existing particles. This contact can be with particles of the same type or with larger particles (mainly in concentrated environment). Coagulation can be the result of gravitation, turbulence and shearing, or, more efficiently,

Brownian motion. These secondary particles formed in this manner have the longest lifetimes. They accumulate for several weeks and transport toxic compounds over considerable distances.

Particle exposure

Smoking is the main and the most studied but not the only risk factor for the COPD development. Occupational exposures to dusts, vapors, gases, and fumes, exposure to indoor and outdoor air pollution were recently acknowledged as COPD risk factors [45,46]. More than 20% of COPD cases are attributable to occupational exposure [45], while for non-smokers, the estimated attributable risk for COPD associated with occupational exposure is 31% [47]. Since both occupational and environmental exposure to particulate matter are suspected in both the development and exacerbation of COPD [48,49] and in the worsening of its prognosis, primary and secondary prevention of COPD is paramount.

Several recent epidemiological studies have demonstrated the adverse health effects of fine particulate matter. In general, studies do not identify a safety threshold or concentration below which they would have no effect (NOAEL) [50-54]. For this reason, the World Health Organization (WHO) does not provide a margin of safety since the toxic effects of particles exists even at modest concentrations. In the vast majority of cases, the linear increase in the particulate load in air is associated with adverse health effects such as COPD.

The WHO's Global Burden of Diseases (GBD) project concluded that 3.2 million people die prematurely every year from illnesses including COPD that are attributable to particulate air pollution; the majority of global deaths 35% of these deaths occurs in East Asia and Pacific [55]. Brauer et al., [56] have reported that 99% of the population in South and East Asia live in area where the WHO Air Quality Guideline (annual average of 5 µg/m^3 (value updated in 2021 [57])) for $PM_{2.5}$ is exceeded. Particulate matter pollution was also ranked ninth of all the risk factors in terms of years lost to disability [56].

Certain occupational categories should also be considered at risk as they are exposed to particulate matter pollution to a greater extent and more frequently than the rest of the population. Many work activities represent a source of particles emission leading to an increased occurrence of health disorders among exposed workers (e.g., welders, construction workers, farmers, underground workers [58-61]).

Situation of workers in railway enclosures

Several studies have been carried out within underground railway enclosures (EFS) showing much higher PM_{10} concentrations than those measured in outdoor air [62,63]. The main

sources of emissions of these particles are material wear due to wheel-brake friction, followed by wheel-rail contact and contact between rolling stock and the power supply system. Other potential sources for the emission of these particles are diesel engines during maintenance operations, track ballast (composed of silica) used during emergency braking or hillside maneuvers to increase wheel adhesion to the rails, and the supply of outside air. In addition, tunnels are rather dry and difficult to clean, which contribute to resuspension of fine particles when people or vehicles pass through or by forced ventilation or draughts. Due to their origin, the physicochemical composition and own toxicity differ from particles in outdoor urban air. The underground subway particles are mainly composed of submicronic particles, of variable shapes, flaky, semi-spherical, and ellipsoidal, in contrast to the rather spherical combustion particles [64].

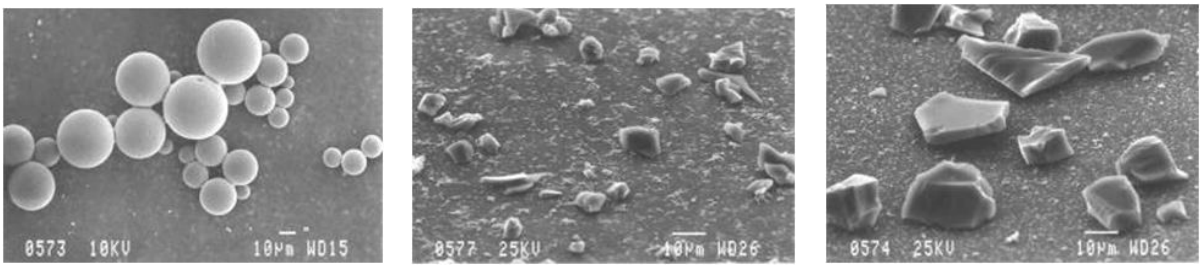


Figure 4. *Different aerosols with different sizes and shapes*

These particles are mainly composed of metals such as iron [65] in metal and oxides form (hematite, magnetite), elemental carbon and organic carbon. Other chemical pollutants may be present such as aromatic hydrocarbons (toluene, phenanthrene, fluoranthene, anthracene and pyrene), and at lower concentrations benzene, nitrogen dioxide and benzo(a)pyrene [66,67]. Some aromatic hydrocarbons are more studied than others, either because of health impact such as Benzo(*a*)pyrene identified as carcinogen (group 1) by the International Agency for Research on Cancer (IARC); or because they are more representative of cumulative exposure to polycyclic aromatic hydrocarbon such as hydroxypyrene.

The PM_{10} concentration measured on the subway platform increases most often between 2 AM and 5 AM, where during this period, diesel engines may operate and infrastructure maintenance work may be carried out [65]. These maintenance operations can be more inconvenient as they take place in a confined space.

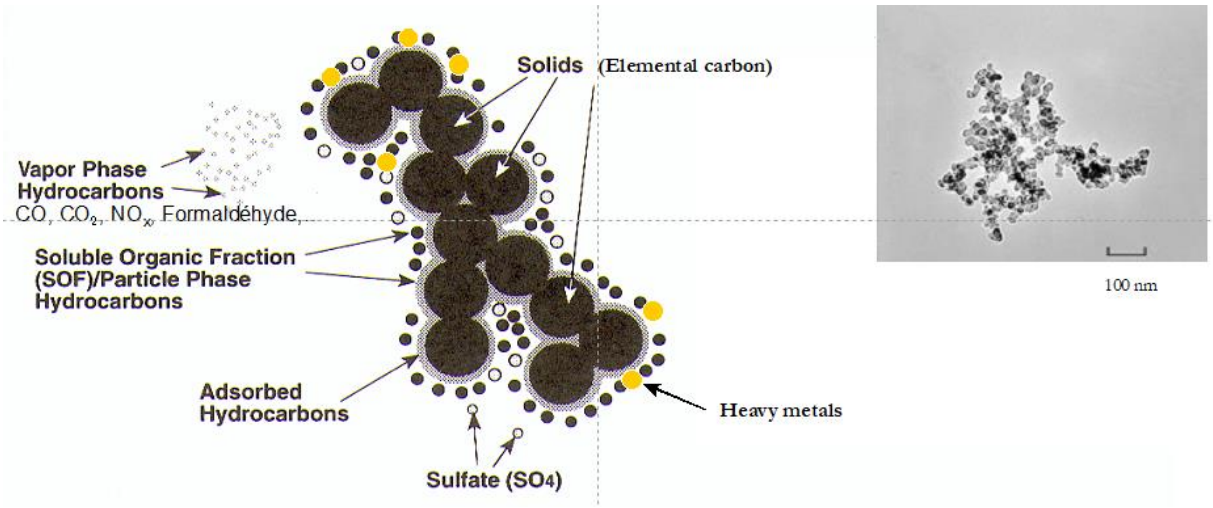


Figure 5. *Agglomerated particle of Diesel [68]*

To date, the toxicity of these suspended particles characteristic of EFS on workers in railway enclosures is not yet known [69,70]. However, by analogy with studies on chronic exposure to particles matter in outdoor ambient air, adverse respiratory health effects can be expected from chronic exposure to EFS particles. The *Conseil supérieur d'hygiène publique de France* recommends for users a reference value of 347 µg/m^3 [71] for PM_{10} based on a daily two-hour presence in EFS. However, considering the duration of attendance by workers exercising their professional activity in the EFS, and the absence of a regulatory standard, it cannot be excluded that particulate pollution in the EFS may have an impact on the health of EFS workers.

From an epidemiological point of view, the data available to date on workers in EFS and on the toxicity of airborne particles in EFS do not enable to conclude on the long-term risks, given the limited number of studies and particularly the issues in the exposure assessment [72-74]. The diversity of workplaces (e.g., underground platforms, trains, corridors) and their surrounding environment (e.g., active ventilation, subway cabin heater) are not taken into account in the studies. For example, the occupation of locomotive operator could be one of the most exposed to high PM_{10} concentrations [65]. This excess of exposure compared to other subway occupations could be due to their constant presence in the tunnels, in unfiltered air cabins with no air-conditioning and whose windows can remain open. Part of their work also involves small maintenance activities in tunnels terminus, which could increase their exposure.

Finally, there is no data on possible excess mortality due to air pollution in underground railway enclosures. Some studies that have been conducted [65] did not report significant effects on respiratory health, but did not discard potential risks associated to the EFS air quality.

Particulate matter toxicological mechanisms

The mechanisms by which airborne particles interact and give rise to adverse health effects as COPD are not clearly understood [72]. Using *in vitro* studies, Seaton et al., [75] have shown that inhaled particles induce the secretion of certain inflammatory mediators by both macrophages and respiratory epithelial cells through complex mechanisms, activating cascades of molecular events. In a study on fine particle ($PM_{2.5}$), Lindbom et al., [76] showed that exposure of human type II pneumocytes (cell line A549) to increasing concentrations (1-100 µg/mL) of $PM_{2.5}$ induced dose-dependent IL-8 secretion with cytotoxicity at the highest dose. Murine macrophages (cell line RAW 264.7) exposed to increasing concentrations (i.e., from 1 to 100 µg/mL) of PM_{10} particles from the Stockholm subway showed to cause significant secretion of TNF-alpha and IL-6. This cascade of pro-inflammatory molecular events can be activated in different ways but it is noted that it may be the consequence of the very high pro-oxidant potential of some particles such as those emitted in EFS. They may have the capacity to generate oxidative stress (OS) and to lead to the activation of transcription factors involved in the regulation of the expression of pro-inflammatory cytokine and chemokine genes [77].

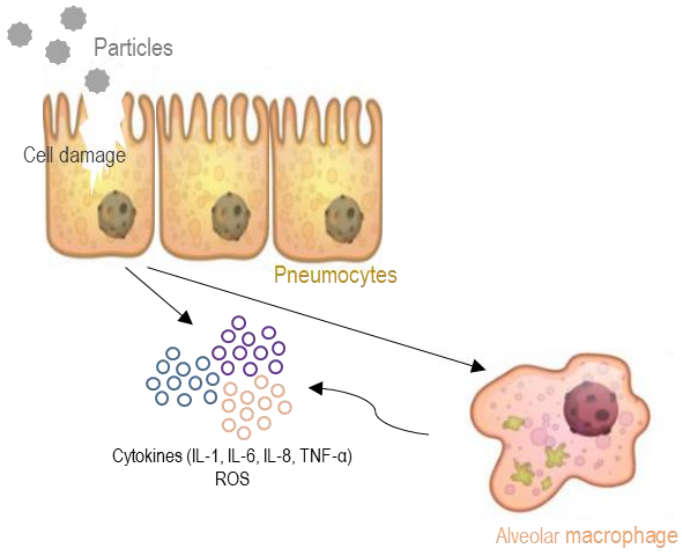


Figure 6. *Illustration of particles-induced oxidative stress and inflammation in pneumocytes and macrophages*

Nevertheless, this is not the only possible effect of the particles: they can also contribute to the formation of free radicals such as the hydroxyl radical. These radicals cause oxidative damage to lipids, proteins and nucleic acids responsible for cytotoxicity and genotoxicity. Karlsson et al., [78,79] characterized the genotoxic potential of PM_{10} particles collected from the Stockholm metro on a type II pneumocyte line (A549). After 4 hours of exposure, an increase in deoxyribonucleic acid (DNA) breakage was observed, depending on the particle concentration.

These genotoxic effects are associated with a rapid increase in the production of Reactive oxygen species (ROS) in the cells after two hours of exposure.
OS can also play a pivotal role in the pathogenesis of COPD particularly in amplifying existing lung inflammation. ROS will alter remodeling of extracellular matrix and blood vessels, stimulate mucus secretion (resulting in a chronic productive cough) and inactivate anti-proteases responsible of many antioxidant pathways. These damages will finally conduct to hyper-responsiveness of peripheral airways [80].

Particles may also inhibit the anti-inflammatory capacity of certain proteins. Araujo et al., [81] reported that in animal studies, UFPs induce the inhibition of the anti-inflammatory capacity of high-density lipoprotein and in greater systemic OS, as evidenced by a significant increase in hepatic malondialdehyde (MDA) levels and upregulation of transcription nuclear factor erythroid 2 related factor 2 (Nrf-2)-regulated antioxidant genes.

Many toxicological studies have been undertaken with animals and *in vitro* protocols and clearly demonstrated that, for particles with the same chemical composition, small particles are more hazardous than larger ones [64,82,83]. This negative effect of UFPs is due to their higher surface-to-volume ratio [84] and their particle surface characteristics that appear to play a key-role in interactions with cells and organs [85] and in the formation of ROS [86]. Brown et al., [87] suggested that this is one of the ways in which low toxicity particles such as UFP induce adverse health effects resulting in pro-inflammatory activity as a consequence of their large surface area. Karlsson et al., [88] demonstrated that the genotoxicity of PM_{10} particles in the Stockholm metro is associated with a surface reactivity of these particles rich in various metallic elements, especially iron. Similarly, Kam et al., [89] consider that the specific content of particles in various elements, particularly soluble iron, present in the particles is sufficient to predict nearly 94% of the production of OS induced by these particles and their inflammatory response. Nevertheless, despite these data there are currently no studies that determine a clear association between UFP and COPD.

3. Current diagnosis of chronic obstructive pulmonary disease

An early diagnosis and appropriate treatment of COPD are critical to decrease the rate of mortality. In 1998, a Global Initiative for Chronic Obstructive Lung Disease (GOLD) program was initiated through a concerted worldwide effort of people involved in all facets of healthcare and healthcare policy. Its aim was to provide recommendations based on the scientific literature for the prevention, assessment, diagnosis and treatment of patients with COPD that could supplement to the work of the clinician. These recommendations have as an eventual

objective directed towards relieving and reducing the impact of symptoms and the risk of adverse health effects as exacerbations events that may affect the patient.

The assessment of COPD takes place in several steps in order to determine the presence and severity of airflow limitation, the magnitude of the patient's symptoms, the future risk of events (such as exacerbations, hospital admissions or death).

Presence of airflow limitation

Currently, spirometry is required to make the diagnosis of airflow limitation in clinical context. Spirometry measures the volume or air that the patient can forcibly expel from the lung after a maximal inspiration (forced vital capacity, FVC) and the volume of air exhaled during the first second of forced expiration. (Forced expiratory volume in one second, FEV_1), and the ratio of these two measurements (FEV_1/FVC). Spirometry measure is evaluated by comparison with reference values based on sex, age, height, weight and ethnicity [90]. In case of a FEV_1/FVC ratio measured below the lower limit of the predicted normal range (< 70%), a reversibility test should be performed 15 min after administering bronchodilator. The presence of a post-bronchodilator FEV_1/FVC ratio < 70% confirms the presence of persistent airflow limitation in presence of the clinical symptoms such as dyspnea, chronic cough or sputum production.

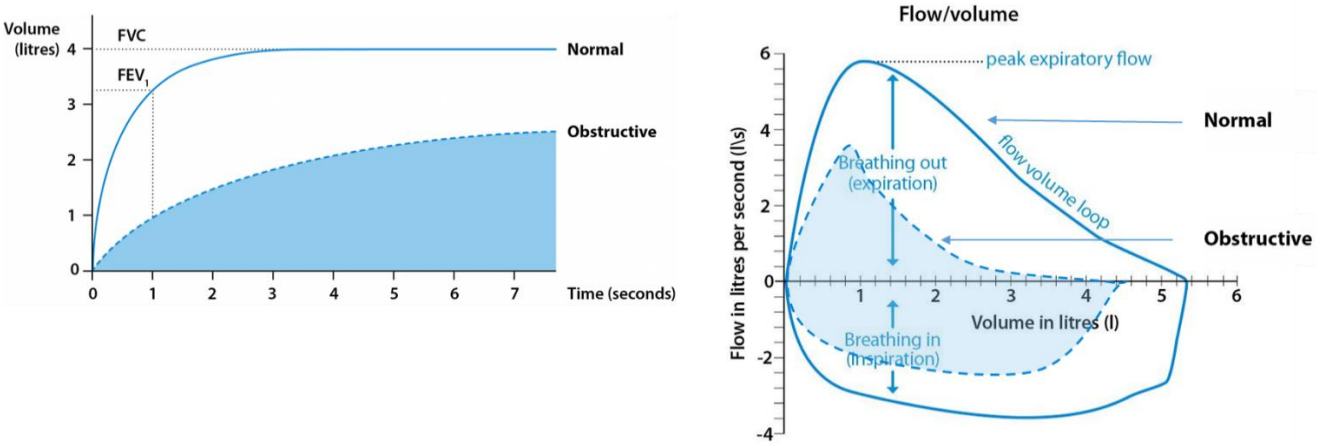


Figure 7. *Spirometry testing for differentiating obstructive from normal pattern. Illustration modified from [91]*

Severity of airflow limitation

In case of positivity of spirometry test, the clinician will diagnose this airflow limitation as COPD and its severity will be classified according to the percentage of FEV_1 on the predicted values for that individual. The classification is summarized in the Table 1.

Table 1. *Classification of airflow limitation severity in COPD in patients with $FEV_1/FVC < 70\%$*

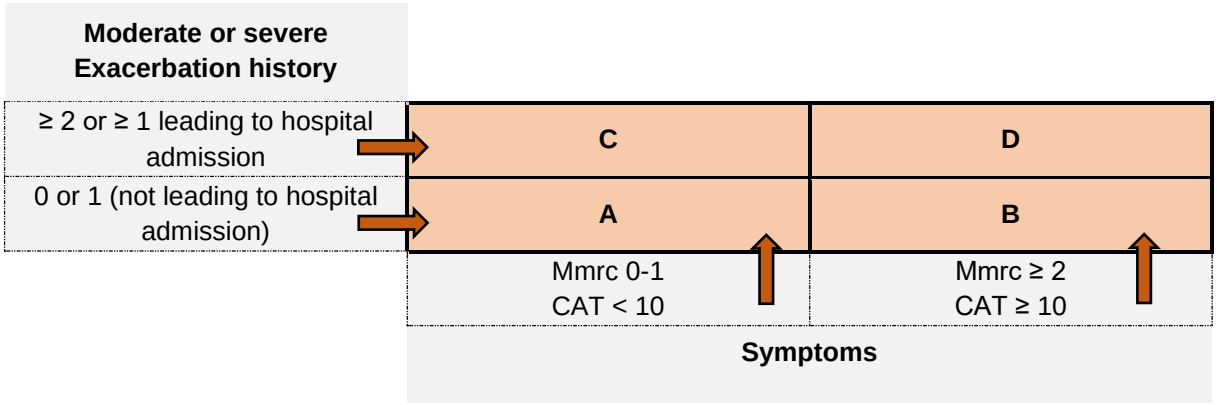
		AFTER BRONCHODILATOR
GOLD 1	Mild	$FEV_1 \geq 80\%$ predicted
GOLD 2	Moderate	$50\% \leq FEV_1 < 80\%$ predicted
GOLD 3	Severe	$30\% \leq FEV_1 < 50\%$ predicted
GOLD 4	Very severe	$FEV_1 < 30\%$ predicted

Relatively recent studies have suggested that there was significant relationship between the decrease in respiratory function measured by spirometry with the risk of exacerbations of respiratory symptoms (number and severity) hospitalization [92] and death [93]. Exacerbations become more frequent and severe for patients with severe COPD compared to patient with mild to moderate COPD where the rate of COPD exacerbations may increase to 0.5 to 2 /patient/year [94]. Proportionally, approximately 20% of GOLD 2 patients will experiment frequent exacerbations requiring treatment with antibiotics and/or systemic corticosteroids and hospitalization, while in GOLD 4 patients, this number increases to 50% [94].

The magnitude of the patient's symptoms and assessment of exacerbation risk

To measure the magnitude of symptoms and assessment of exacerbation risk, the patient will be assessed for dyspnea using simple and reliable questionnaires designed for use in routine daily clinical practice such as mMRC (Modified British Medical Research Council) [95] or symptoms using COPD Assessment Test (CAT) – for a measure of health impairment in COPD [96]. CAT questionnaire consists of eight items selected to cover a wide range of disease severities: cough, phlegm, chest tightness, breathlessness going up hills/stairs, activity limitation at home, confidence leaving home, sleep and energy. Then, the history of moderate and severe exacerbations, including previous hospitalizations are recorded. Exacerbations events will be classified as mild (bronchodilators), moderate (bronchodilators + antibiotics/ oral corticosteroids) or severe (requiring hospital treatment) [97]. Severe exacerbations will be associated with acute respiratory failure, will have a negative impact on patient prognosis with an increased risk of death [28]. Finally, all these information (symptom burden and risk of exacerbation) are compiled via an ABCD system (Table 2) which can be used to guide therapy.

Table 2. *ABCD system for assessment of symptoms/risk of exacerbations*



For example: A patient with history of > 3 moderate exacerbations in the past year, CAT score of 15 would be classified as Group D. mMRC =Modified British Medical Research Council; CAT = COPD Assessment Test.

4. Limitations in the current diagnostic approach

Spirometry is the only current clinically approved and reproducible test to evaluate lung function, to monitor disease progression, severity and response to therapy. Nevertheless, spirometry does not have any prognostic value [98] and is still uninformative with respect to the prediction of the progression, exacerbation of the disease and whether the disease has been present for many years. Moreover, COPD also exist in individuals with normal spirometry [99]. It is also a procedure that cannot be performed in every location because it requires specific equipment, medical training and a supervised drug administration. The results may vary depending on the nurse or doctors performing the test but also on the patient's compliance [100,101].

Therefore, **biomarkers** that can be used for early COPD diagnosis or for identifying people susceptible to COPD, for exacerbation prognosis, for predicting the response to therapy and for obtaining efficient stratification of COPD patients for personalized treatment. These personalized treatments will aid physicians to treat patients more efficiently, allowing better chances of survival and/or quality of life.

II. Emerging biomarkers in chronic obstructive pulmonary disease diagnosis

1. Definition and typology of biomarkers

The United Nations Environment Programme led by the International Labour Organization (ILO) and WHO, and carried out under the Inter-Organization Program for the Rational Management of Chemicals, has defined a biomarker as "any substance, structure or process that can be measured in the body or its products and that influences or predicts the incidence of an outcome or disease" [102]. This definition takes into account unintended exposure to the environment, e.g., chemicals, as well as incidence (prediction/diagnosis), outcome of disease, but also the effects of treatments and interventions (progression/regression).

It also includes, according to the WHO "…any measure that reflects an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" [103]. Biomarkers are only those quantifiable medical signs that can be measured in a reproducible manner. They are indicators of changes and adverse effects in biological systems, while it is often impossible to access the target organ of biological effect [104,105].
Their use, especially in laboratory and clinical research, must be closely related to relevant clinical reference parameters without substituting or replacing them as they may not be part of the pathophysiological pathway leading to this outcome (e.g., in case of interdependent diseases [106]). Thus, biomarkers prematurely accepted as surrogates for clinical endpoints may pose substantial risks and lead to erroneous research conclusions [106]. This has already occurred in a Cardiac Arrhythmia Suppression Trial (CAST) that used antiarrhythmic drugs to decrease morbidity from cardiovascular disease. Drugs such as flecainide were used to suppress "arrhythmia" considered the surrogate biomarker for "myocardial infarction" but, conversely, this increased mortality in some patient populations [107,108].

Biomarkers can be classified into three main categories [109,110] based on the sequence of events from exposure to disease (Figure 8):
- Exposure biomarkers;
- Biomarkers of effect;
- Biomarkers of susceptibility.

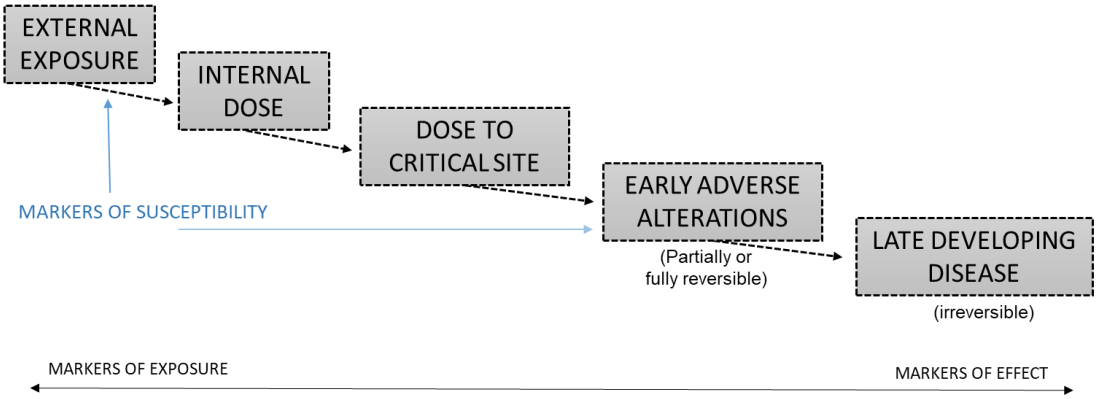


Figure 8. *Biomarkers of exposure, effect and susceptibility modified from* [111]

Although different types of biomarkers are considered distinct for classification purposes, it is not always possible to assign them with certainty to a single category. This depends on the toxicological significance and the context in which they are measured.

Susceptibility biomarkers can reflect variations in exposure, kinetics, and effects. They can lead to both a different effect of an environmental exposure on disease risk in individuals with different genotypes and a different disease risk on in individuals with the same environmental exposure [112].

An exposure biomarker is defined as "*an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and a target molecule or cell that is measured in a compartment within an organism*" [113]. As indicators of chemical uptake in the body, exposure biomarkers can be useful in industrial toxicology and occupational medicine for more accurate risk assessment and internal dose modeling. A good exposure biomarker should be useful for predicting adverse effects, rather than exposure levels. This may be particularly the case when accurate and valid measurements of "true" exposure are difficult or impossible to obtain (e.g., use of protective devices, multiple routes of absorption) [102]. For example, chronic exposure to organochlorines is better indicated by serum organochlorine levels than by industrial hygiene measures. Metals from subway airborne dust and subsequently found in urine generally reflect short-term exposure unless where their biological half-life in body is particularly long [114].

A biomarker of effect can be defined as "any measurable biochemical, structural, functional, behavioral, or other type of alteration in an organism that, depending on its magnitude, may be associated with an established or potential impairment of health or disease" [113].

A good biomarker of effect is one that is highly predictive of clinical disease, i.e., disease never or rarely occurs without this indicator. This can be assessed by calculating the proportion attributable to a particular biomarker and estimating the proportion of disease cases that must progress because of the biomarker or that would not occur if the event(s) causing it could be prevented [115]. This proportion takes into account the strength of the association between the marker and the disease and the prevalence of the marker. Second, the biomarker of effect must be able to reflect the relationship between an exposure and the outcome (a disease) as well as to provide a high positive predictive value by discriminating in a population the proportion of individuals with a particular disease among all individuals who have the given level of the biomarker tested [102]. Finally, it could also reflect early biological signs following exposures that preferably would be reversible (decrease or disappear upon removal of the exposure).
Biomarkers of effect are mainly used in health surveillance or early disease detection programs to identify populations at risk and to implement preventive measures. For example, with the early detection of patients who might develop diabetes mellitus by measuring blood glucose concentration (e.g., at 1.26 g/L in the morning on an empty stomach or above 2 g/L during the day) and quickly implementing preventive measures (weight loss, physical activity and dietary changes). Another example is the use of the biomarker of effect as a stratification tool (e.g., GLEASON Score [116,117]) or for classification of the extent, growth and metastasis of prostate cancer by measuring the concentration of prostate specific antigen (PSA) in the blood.

A susceptibility biomarker can be defined as "an indicator of an inherent or acquired capacity of an organism to respond to chemical exposure" [105,118]. Inherent capacity includes genetic polymorphisms of enzymes that metabolize drugs or that influence/control cell differentiation, apoptosis, cell cycle kinetics, signal transduction, and DNA repair. These biomarkers can be used to distinguish populations based on their degree of sensitivity to exposure. For example, a susceptibility biomarker will allow a population to be stratified based on a specific "genotype" associated with a disease rather than relying on a report of family history of its disease. The ability to quantify "susceptibility" in this manner may provide an extremely valuable method for estimating disease risk among diverse populations with similar levels of exposure and focusing prevention and control programs on high-risk individuals. For example, individuals deficient in glucose-6-phosphate dehydrogenase are more susceptible to toxic oxidants present in the environment [119].

Biomarkers are required in several scientific fields. They are useful in epidemiology because of the need for objective means to quantify exposures to specific agents. Clinicians need to rely on clinical signs and identify early-stage biomarkers of disease processes to guide their

diagnoses and treatment regimens. In this way, appropriate therapeutic and preventive measures can be put in place to halt disease progression. Toxicologists are interested in exposure biomarkers and effect biomarkers in animal and human studies. They can be measured for any event from exposure to a toxic chemical to the development of pathology. However, the mechanism of a chemically induced disease must be known in order to assess the dose at the site of interest and be related to the early adverse alterations that precede the development of a disease state. When the exact mechanism is not known, reliance should be placed on exposure biomarkers that indicate the total internal dose received by an individual that is related to early adverse alterations or development of a disease state [111].
Some biomarkers can serve as alternatives to the use of pharmacokinetic models for the purpose of determining actual exposure and internal dose. Because of extraordinary sensitivity, they can be measured at dose levels well below those at which effects are directly observable. For example, measurements of biomarkers from oxidative stress can be used to indicate the risk of developing lung disease [120].

Ideally, biomarkers should be accessible (i.e., available by non-invasive techniques), non-destructive, inexpensive to test and easy to measure. The ideal biomarker for exposure measurement is a chemical marker that is specific, sensitive (with the ability to distinguish between populations with different exposure levels, susceptibilities, or degrees of effect), and detectable at trace levels [121]. For effect biomarkers, they must have the ability to account for all or most of the variation in a physiological state, be highly specific for the effect in question, have a well-established relationship between the biomarker response and the damage induced, and finally have a low background level in the body fluid in question. Few, if any, biomarkers have all of these characteristics, but many non-ideal biomarkers may be useful for specific purposes [111].

2. Constraints in the implementation of new biomarkers

Before implementing biomarkers in the clinical practice, for instance, with respect to COPD early detection, numerous tests are necessary.
The process from discovery of a biomarker to clinical implementation is usually divided into three main phases (Figure 9):

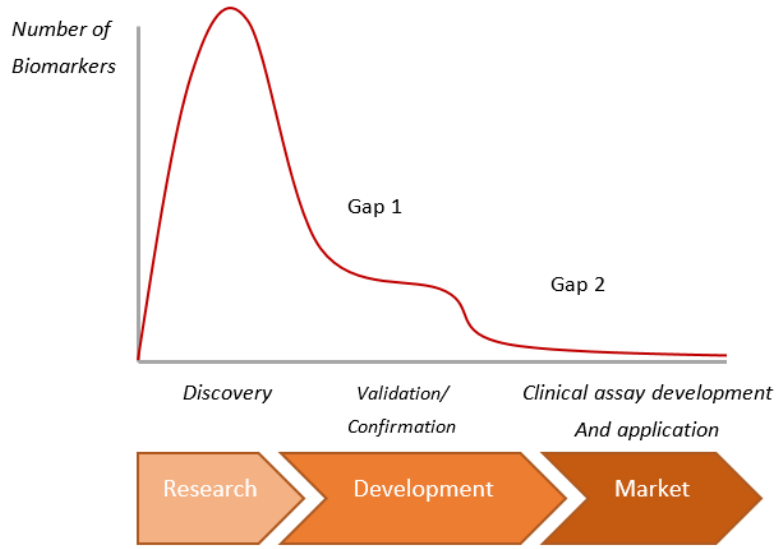


Figure 9. *The number of biomarkers from discovery to clinical use*

The **research** involves discovery studies to identify potential biomarkers for the disease indication. During **development**, the feasibility of the biomarker will be determined through analytical development, method validation and confirmation in clinical and epidemiological studies. Finally, further development and extensive assays will be conducted to obtain a clinical-grade biomarker fulfilling diagnostic and regulatory criteria for **market**. Nevertheless, significant hurdles can occur between the different phases resulting in a drastic decline (gap 1 and gap 2 in Figure 9) in biomarkers accepted by the regulatory authorities for implementation in clinical practice. At this point, many identified biomarkers during the research part are abandoned because of important pre-and post-analytical variabilities. These variabilities are partly the result of the current way of operating of different research groups in academia that use non-compliant and various detection, calibration, and validation and statistic methods or poorly reported analytical protocols and study designs. This way of operating leads to the inaccurate detection of biomarkers, the irreproducibility of studies determining the relevance of biomarkers to clinical practice and the discrepancy between studies performed by academia and studies required for regulatory purposes [122]. Eventually, if the identified biomarker reaches the clinical trial stage, its feasibility will be subject to a certain skepticism and sometimes even an outright negative attitude toward performing such trials at all. Laboratories and companies will be thus forced to investing significant amounts of money into the reevaluation of the biomarker. For this reason, consistent efforts for standardization and harmonization of methods must be undertaken on behalf of academia researchers to regain the confidence of companies and laboratories in the specific biomarker with respect to the regulatory authorities.

In this sense, an EU funded consortium CliniMARK [123] was formed to increase clinically validated biomarkers by establishing ‘Best Biomarker Practice’ guidelines, having COPD as

disease demonstrator. Its aim is: I) to significantly increase the number of clinically validated biomarkers by means of time-efficient biomarker detection studies; II) to improve the quality and reproducibility of clinical feasibility studies; III) to improve the quality standard of published analytical methods (with regards to sample stability, specific matrix effects, expected assay performance); IV) to standardize data deposition and increase of the effectiveness and comparability of current research efforts. With regard to COPD, CliniMARK network gathered and classified information on the most promising biomarkers highlighted during discovery experiments with the indication of the diagnosis, companion diagnostic or prognosis for COPD and selected the appropriate research-grade detection tests for their translation toward market phase [124,125].

3. Oxidative stress in chronic obstructive pulmonary disease

Within CliniMARK network, our work has focused on the standardization of new analytical methods of oxidative stress biomarkers relevant for the COPD detection. These methods are based on OS that could be measured in non-invasively collected matrices, enabling repeated measurements in the prospective studies. OS is thought to play a pivotal role in the pathogenesis of COPD particularly in amplifying lung inflammation and subsequent exacerbation in COPD. *(Refer section 2. § Particulate matter toxicological mechanisms)*

The next literature addresses key elements regarding the origin of OS and its physiopathology.

III. Oxidative stress

1. Pathophysiology of oxidative stress

OS is implicated as one of the major underlying mechanisms behind many acute and chronic diseases including COPD [126]. OS is defined as an imbalance state between the continuous production and elimination of ROS occurring in a normal cell metabolism. This metabolism include physiological processes such as regulation of cell redox homeostasis, cell signaling and in the detoxification mechanism by antioxidant enzymes toward the generation of ROS [127].

ROS is a collective term that includes both non-radicals that are oxidizing agents and/or are easily converted into radicals (HOCl, HOBr, O_3, $ONOO^-$, O_2, H_2O_2) and oxygen-derived free radicals (Table 3). Free radicals can be defined as molecules containing one or more unpaired electrons in atomic or molecular orbitals. This unpaired electron gives them a high degree of reactivity and a special ability to react with the surrounding macromolecules via radical

reactions [128]. The most important oxygen-derived free radicals are superoxide $O_2^{\bullet -}$, hydroxyl $OH^{\bullet}$, peroxyl $RO_2^{\bullet}$ and hydroperoxyl $HO_2^{\bullet}$. Nevertheless, the biological reactivity of these oxygen-derived free radicals proves to be highly variable depending on the radical under consideration. In some cases, at low to moderate concentrations, ROS constitute an essential mechanism for cellular signaling that contributes to maintain the redox homeostasis and vital functions of the cell.

Table 3. *Non-exhaustive list of reactive oxygen species*

Radicals	Non-radicals
Superoxide $\mathbf{O_2^{\bullet -}}$	Hydrogen peroxide $\mathbf{H_2O_2}$
Hydroperoxyl $\mathbf{HO_2^{\bullet}}$	Peroxynitrite $\mathbf{ONOO^-}$
Hydroxyl $\mathbf{OH^{\bullet}}$	Peroxynitrous acid **ONOOH**
Peroxyl $\mathbf{RO_2^{\bullet}}$	Nitrosoperoxycarbonate $\mathbf{ONOOCO2^-}$
Alcoxyl $\mathbf{RO^{\bullet}}$	Hypochlorous acid **HOCl**
Carbonate $\mathbf{CO_3^{\bullet -}}$	Hypobromous acid **HOBr**
Carbone dioxide $\mathbf{CO_2^{\bullet -}}$	Ozone $\mathbf{O_3}$
Singlet $\mathbf{O_2{}^1\sum g^+}$	Singlet oxygen $\mathbf{{}^1\Delta g}$

The rupture of redox homeostasis can result in:

- Mutations affecting the activities of antioxidant defense enzymes such as CuZnSOD, or glutathione peroxidase [129] leading in depletion of antioxidant defenses;
- Excessive activation of 'natural' systems producing reactive species [130].

According to the hierarchical oxidative stress hypothesis [131], low levels of OS induce protective effects. However, at OS levels increase drastically, adverse effects can occur. Protective effects are induced by Nrf-2 (Tier 1 in Figure 10). Nrf-2 regulates the transcriptional activation of more than 200 antioxidants and detoxification enzymes via activation of the promoters of phase II genes and antioxidant response element (ARE) and that would restore cellular redox homeostasis. Defects or aberrancy of this protective response pathway may result in pro-inflammatory (Tier 2) and cytotoxic (Tier 3) effects and increase disease susceptibility. Inflammatory effects are initiated through a reversible oxidation of the neighboring proteins: ROS then act as second messengers in signaling cascades (e.g., mitogen-activated protein kinase (MAPK)), for induction of gene expression and protein synthesis of certain inflammatory cytokines such as TNF-alpha, interleukin-1, beta, 6 and 8, via the activation of Nuclear Kappa Factor B (NF-kB) [132]. Excessive secretion of these cytokines will lead to the induction of inflammatory events. In some cases, these cytokines can help to the reinstatement of cell homeostasis. However, in other cases, their excessive or aberrant secretion turn out to be deleterious and appears as an amplifying factor in the complex

cascade of inflammatory events [133]. When the immunity systems are deficient and/or the production of ROS is too high, they induce damage in cell structures by the non-specific and irreversible oxidation of cellular macromolecules, leading to loss of their function. Many macromolecules such as lipids, proteins and nucleic acids are susceptible to be damaged or modified by ROS. At the very last stage, (Tier 3) also known as toxic oxidative stress, perturbed mitochondria by ROS will release pro-apoptotic factors and inducing programmed cell death.

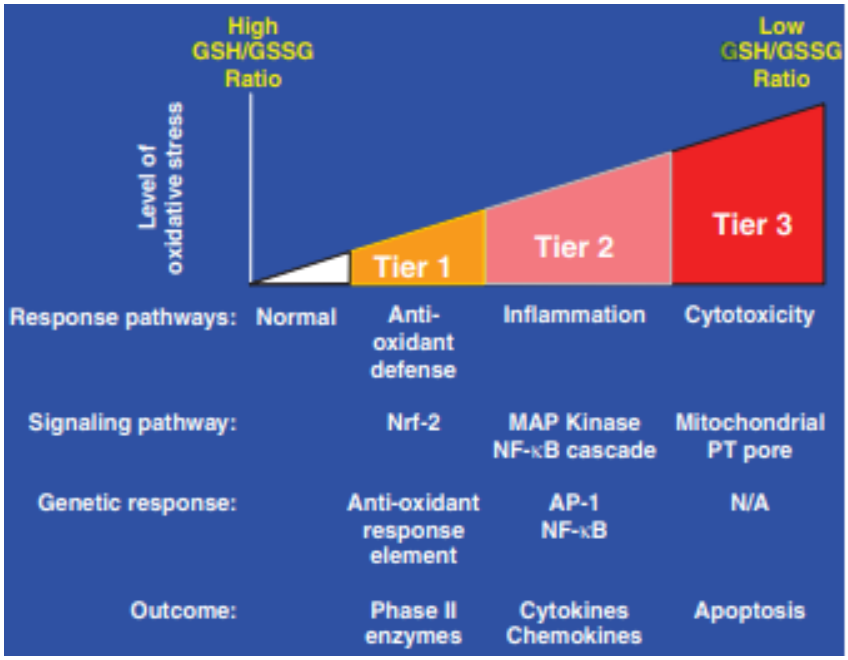


Figure 10. *The hierarchical oxidative stress model from [131]*

2. Reactive oxygen species detection

Despite the growing knowledge regarding the roles of oxidative stress in many serious pathophysiological processes, measurement of ROS is still complicated because free radicals are reactive, *in vivo* short-lived, and of diverse characteristics. Most ROS do not accumulate to sufficiently high levels to be measured directly. There are a few exceptions such a hydrogen peroxide (H_2O_2) [128] which can be measured with a panel of biochemical colorimetric assays. As ROS can oxidize proteins, lipids, and nucleic acids, causing structural and functional cellular changes, ROS activity can be quantified indirectly by measuring oxidized products. The latter can be thus considered as candidate biomarkers of oxidative stress.

Abnormal levels of OS biomarkers are observed in various acute and chronic diseases, e.g., cancer, cardiovascular disease [134], neurodegenerative disease [135], lung disease [120,136,137] and even the normal aging process [138].

It is important to note that the excretion of oxidized products represents the average rate of damage in the total body and includes several elements. For example, the level of oxidized bases in nuclear DNA is a dynamic process and its measurement at a given time reflects the concentration in specific tissues at the moment of sampling [139]. Lesions can direct result from diet (exogenous source) and cell death in addition to DNA repair (endogenous source). When the balance between the organisms' pro-oxidant and antioxidant processes is shifted in favor of the former, oxidative stress increases as well as oxidative DNA damage. However, even in the absence of oxidative stress, some of the radicals escape the antioxidant defenses, resulting in a residual baseline level of damage that, for the most part, does not appear to have any harmful consequences [140].

Biomarkers that could both help in clinical centers to assist physicians in the management of COPD patients and in screening populations at risk to develop COPD, such as occupationally exposed workers or people living in polluted environments (*refer section 2.* § *Situation of workers in railway enclosures).* Biomarkers could be considered as promising alternative methods and would be used in addition to spirometry. Oxidative potential, 8-isoprostane, MDA, and 8-OHdG are all considered as potent, interesting oxidative stress biomarkers, and could be relevant and useful candidates to monitor COPD evolution and severity. Among biomarkers that may associated with COPD diagnosis, these latter can be measured non-invasively.

IV. Oxidative stress biomarkers

Biomarkers of oxidative stress can be classified in different families (DNA, lipids e.g., OxLDL, proteins e.g., S-glutathionylation Nitrotyrosine, carbohydrates). They are produced by interactions with ROS in the microenvironment (e.g., GPX-1 that are molecules of the antioxidant system that change in response to increased oxidative stress). In the following paragraphs, we selected two major types of oxidative molecular targets (lipoperoxidation and nuclear acid oxidation) as they are the most promising biomarkers candidates in relation to COPD.

1. Lipoperoxidation

Lipids can be oxidized, chlorinated and nitrated by a range of ROS excepting H_2O_2, $NO^{\bullet}$ or $O_2^{\bullet -}$, which are essentially unreactive with lipids [141]. The radical degradation of lipids by oxidation, also known as lipid peroxidation is the most investigated processes used for the assessment of oxidative stress in human studies. Lipids in biological membranes and lipoproteins are major peroxidation targets that can be explained by the abundant presence of

membrane phospholipids at sites where radicals in general and, more specifically, ROS are formed. The hydroxyl radical (•OH), the most reactive and hazardous radical, plays an important role in the reactions of lipid peroxidation by oxidizing lipids containing carbon-carbon double bonds, especially polyunsaturated fatty acids (PUFA) [142] such as arachidonic acid and docosahexaenoic acid [143] leading to a radical chain reaction and resulting in the formation of many equivalents of hydroperoxides (ROOH). These subsequently undergoes fragmentation to produce a broad range of relatively stable end products in variable amounts, including alkanes, carbonyl, F2-Isoprostanes and MDA [141]. Some of these products, especially hydroxyalkenals, are toxic by themselves and may serve as second messengers for radical damage. Other products like MDA and unsaturated aldehydes, are capable of inactivating many cellular proteins by forming protein cross-linkages [144].

a. 8-isoprostane

Isoprostanes (IsoPs) are a family of eicosanoids of non-enzymatic origin, mainly derived from arachidonic acid (AA) independent of cyclooxygenase action. Some particular isoprostanes are derived from eicosapentaenoic and docosahexaenoic acids. These are often called neuroprostanes or F4-isoprostanes [145,146].

Isoprostanes are produced by random ROS oxidation of polyunsaturated acyl groups on AA in situ in membrane phospholipids. This step leads to the capture of a hydrogen atom from a carbon positioned between two unsaturated sites on the AA. The reaction occurs at C-7, 10 or C-13 (for F2-isoprostanes). Subsequently, the reaction of the resulting radical with molecular oxygen at C 5-, 8-, 9-, 12- and 15- positions leads to the formation of a cyclopentane ring characteristic of F2-isoprostane. F2-IsoPs have the same molecular formula but have a wider range of structures because their syntheses are more random than those of prostaglandins. A summary of their formation is shown in Figure 11.

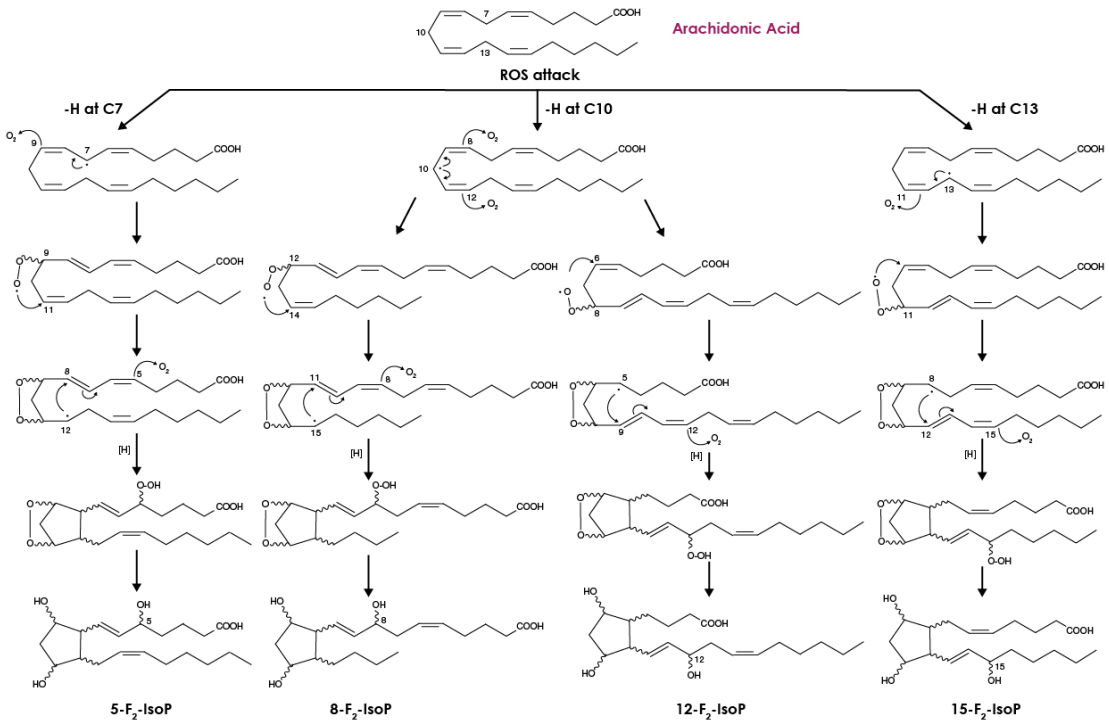


Figure 11. *IsoPs formation from* [147]

There are four series of F2-isoPs present in vivo. Each of the four subfamilies consists of 16 diastereomers, giving a total of 64 F2-isoPs that can be formed during arachidonic acid peroxidation [143,147,148].

The formation of isoprostane from arachidonic acid in phospholipids significantly disrupts biological membranes [149]. Isoprostanes are therefore rapidly excised and exported to the extracellular environment. They are then released into the bloodstream by phospholipases, rapidly metabolised in the liver, and excreted as free acids in the urine.

The 8-isoprostane produced from 13-peroxy radicals, is one of the most extensively studied F2-isoprostanes and the most commonly used biomarker for the assessment of oxidative stress in human studies [150,151]. Although 8-isoprostane can be produced by cyclo-oxygenase-1 and/or cyclo-oxygenase-2 activity in some cells and tissues, both *in vitro* and *in vivo*, its production in humans is mainly derived from lipoperoxidation due to ROS. For this reason, measurement of 8-isoprostane levels in biological fluids has been considered an ideal method of quantifying oxidative stress in different pathophysiological conditions.

8-isoprostane has been shown to have biological activity: it is a potent pulmonary and renal vasoconstrictor [152] and has been implicated as a causative mediator of hepato-renal syndrome and pulmonary oxygen toxicity [153]. 8-Isoprostane has been proposed as a marker for antioxidant deficiency and oxidative stress and elevated levels have been found in heavy

smokers. 8-isoprostane levels are also a relative indicator of sample integrity for lipid-containing samples such as serum, plasma, and whole cell preparations. Plasma from healthy volunteers contains modest amounts of 8-isoprostane (40-100 pg/ml) that increase with the age of the test subject. Normal human urinary levels of 8-isoprostane range from 10-50 ng/mmol (creatinine normalized) [154,155], which is an order of magnitude higher than many enzymatically derived eicosanoids [156]. Measurement of 8-isoprostane in plasma reflects systemic oxidative stress, whereas its measurement in the exhaled breath condensate (EBC) is more likely to reflect lipoperoxidation that occurs in the lungs. 8-isoprostane has been measured in EBC in both healthy individuals and in patients with various respiratory tract diseases and is considered one of the most reliable markers of lipid peroxidation and thus oxidant stress status *in vivo* [157].

b. Malondialdehyde

The process of lipid peroxidation induces the formation of peroxyl radicals (ROO•) and then, following rearrangement by cyclisation, the formation of endoperoxides (ROOR'), precursors of MDA. The reaction is shown in Figure 12.

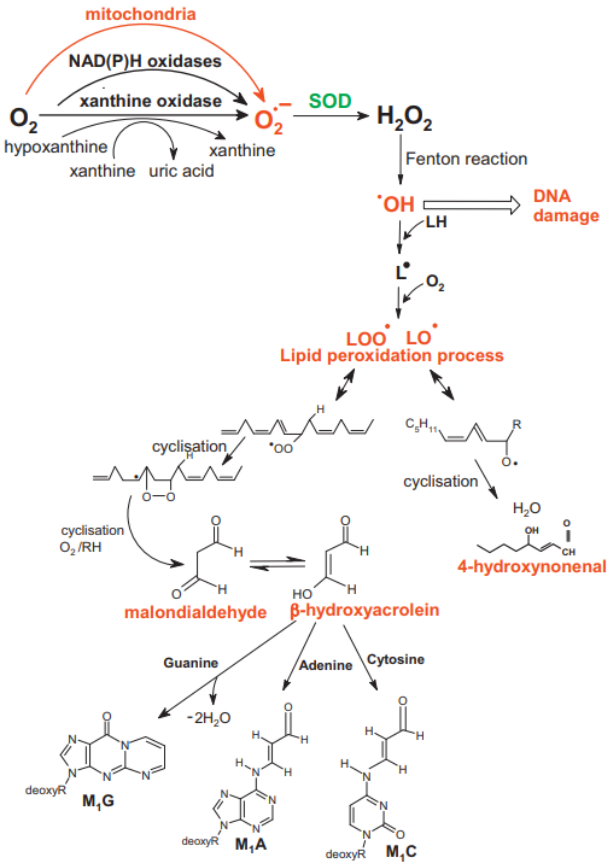


Figure 12. *Formation of malondialdehyde [158]*

MDA play a role in the toxic effects of lipid peroxidation. Aldehyde toxicity is based on the alterations of several cell functions, which mostly depend on the formation of oxidized covalent adducts with the -SH and -NH_2 groups of cellular proteins [143,159] and nucleic acids. It has been reported that the overproduction of bioactive aldehydes results in protein carbonylation associated with a spectrum of disorders including atherosclerosis, diabetes, neurodegenerative diseases and liver disease [160]. For example, MDA has been reported to react *in vivo* with protein-bound lysine residues to form dihydropyridine-type adducts including DHP-lysine [161].

Oxidized adducts were also found in Apo B fractions of oxidized low-density lipoproteins (LDL). Such compromised LDL is removed by macrophages which become fat-filled foam cells, ultimately causing their death. The lipid released is then deposited in the arterial intima as seen in the early stages of atheroma [162,163]. MDA can also interact with DNA bases to form oxidative adducts, enhancing the mutagenic and even carcinogenic potential of oxidative stress [164].

The methods to measure MDA can be divided into two categories: i) assays based on measurements of aldehyde-protein adducts (conjugated MDA) and ii) assays based on determination of the unconjugated form (free MDA) [165].
The sum of unconjugated and conjugated MDA is often defined as total MDA. Free MDA is more commonly used than total MDA as a biomarker of oxidative stress, due to methodological convenience. Total MDA analysis requires additional hydrolysis steps at an elevated temperature to release conjugated MDA into free MDA. Furthermore, this hydrolysis procedure may introduce artifacts through the release of additional MDA molecules from compounds other than MDA-conjugates. Nevertheless, in measuring free MDA, the total amount of MDA generated from lipid peroxidation might be missed. On the other hand, dietary intake can contribute to conjugated MDA since these adducts may be present in foods, for example, after cooking [128].

The concentrations of free MDA and total MDA in EBC and in urine were significantly correlated with each other [165]. In EBC the relationship between free and total MDA is well correlated (R^2 =0.61) [165]. For this reason, using either free or total MDA in EBC may reflect similar oxidative stress levels.

2. Nucleic acid oxidation

ROS are scavenged by the antioxidant system, but when their concentration is too high to be balanced, an oxidative damage to proteins, lipids and DNA is unavoidable [166]. Among the cellular macromolecules affected by oxidative stress, DNA is of crucial importance because it is the repository of genetic information, is present in only one copy in each cell and it is the main target for the induction of cancer [167,168]. A broad range of DNA products are produced during oxidative damage to DNA (damage to deoxyribose phosphate backbone, specific chemical modifications of purine and pyrimidine base, covalent cross-links between DNA and proteins (e.g., MDA and DNA), single- and double strand breaks [169]. Oxidative base modifications may result in mutations, whereas oxidation of deoxyribose moieties may induce base release or DNA strand breaks [170]. Guanine is most easily affected to oxidative attack among the DNA nucleobase, since it possesses the lowest oxidation potential of the four bases [171].

The 8-hydroxy-2-deoxyguanosine (8-OHdG) is generated consecutively to the repair of damage of guanine in DNA. It is one of the most recognized biomarkers of oxidative DNA damage and therefore the most studied biomarker of oxidative DNA damage. *In vivo*, DNA damages are usually repaired by a specific system constituted of endonucleases and glycosylases and the oxidized products are excreted in urine without any further metabolism [172].

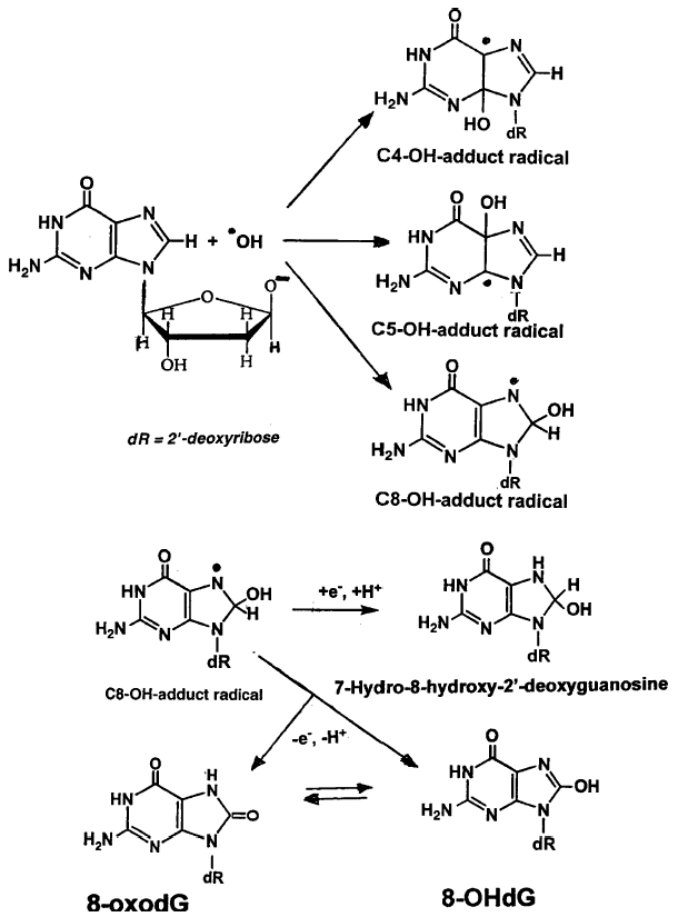


Figure 13. *Reaction of 2'-deoxyguanosine with hydroxyl radicals [173]*

V. Matrices for biomarker analysis

The most commonly used matrices for analysis of oxidative stress biomarkers are blood and urine, others include tears, saliva, broncho-alveolar lavage, sputum and EBC. Blood usually represented an ideal matrix for many chemicals due to its interaction with the organism and its chemical equilibrium with organs and tissues. However, blood has an important disadvantage of requiring an invasive sampling and being challenging to use in occupational or environmental settings for oxidative stress measurement. In this chapter, we focus on the matrices of particular interest for measuring the biomarkers describes in the previous chapter.

1. Urine

Urine appears as a more suitable matrix, with reduced sample manipulation - no severe pre-treatment or pre-purification steps, and thus a reduced risk of pre-analytical artifacts compared to blood [174]. The main advantages of urine as a biological matrix are that it is non-invasive, easy to collect and the matrix is available in abundance. Biomarker concentrations in urine may vary with the volume excreted (depending on the degree of hydration). It is possible to select urine at a specific time of the day to reduce these variations. The first-void urine is usually the most interesting - it is more concentrated because urine has accumulated in the bladder during the night - it is collected before breakfast or any other exposure (including smoking). However, it increases the presence of interfering peaks in urine samples and it becomes a major bugbear - sample ‘cleanup' techniques are critical.

Moreover, measurement in urine is used to reflect overall oxidative damage [175]. Urine provides no information about tissue origin of oxidative damage.

2. Exhaled breath condensate

a. General information

EBC has been developed as a novel and promising non-invasive technique for determining the composition of airway lining fluid in order to directly assess inflammatory and biochemical components of lung diseases as COPD. EBC consists of a gaseous phase containing volatile mediators, and a liquid phase containing non-volatile mediators. EBC is obtained by cooling exhaled breath through contact with a cold surface or condensing collection device. Samples are collected as fluid or frozen material and analysed - immediately or a posteriori - for volatile and non-volatile macromolecules.

EBC can provide complementary information to BAL or sputum and may be useful in clinical diagnostics. Furthermore, BAL or sputum, the number of interfering substances in EBC is significantly low. Indeed, this liquid consists of broncho-alveolar lining fluid (BALF) (< 0.1%) and condensed water (> 99.9%). Very low concentrations of solutes are then present in droplets released from the fluid lining the respiratory membranes. Among the molecules, range of non-volatile compounds is from small inorganic ions (anions, cations), through larger organic molecules (urea, organic acids, amino acids and their derivatives) to peptides, proteins, surfactants and macromolecules. Most inflammatory mediators and other solutes of interest

as oxidative biomarkers are not volatile and are not present in the gaseous phase of exhaled air. Increased concentrations of these biomarkers have been reported in the EBC collected from patients with a variety of pulmonary disorders.

Table 4. *Non-exhaustive list of analytes present in the EBC*

Arachidonic acid metabolites	Leukotrienes B4, C4, D4, E4, prostaglandin E2, cyclopentenone prostaglandins, isoprostanes, thromboxanes
Proteins*	ECP/ EPX, interleukins, TNF Alpha, tumor markers: SCC, TPA-M, CYFRA
Vasoactive peptide amines	acetylcholine, catecholamine, histamine
Serotonin	
Nucleic acids	DNA, RNA, microRNA
Other substances	Thiobarbituric acid reactive substances (TBARS), hydrogen peroxide, nitrate/nitrite, nitrotyrosine, urea, chloride

**Total protein content in EBC is generally in the µg/mL range. Scheideler et al., [176] found protein concentrations between 0.76 µg/mL and 107.7 µg/mL in the condensate of eight healthy individuals.*

b. Collection of EBC samples

EBC sampling is relatively easy, has no side effects unlike BAL [177], which can induce local inflammation, injury or introduce foreign substances into the lung causing infection [177]. It also poses no risk to the patient even if he is under mechanical ventilation and can be repeated frequently [178]. It is a relatively simple, noninvasive procedure in which a person breathes into a sampling device, usually equipped with a mouthpiece, a separator valve, and a condensation tube cooled to below -5 °C, on the walls of which water vapor containing dissolved compounds condenses and is eventually collected for analysis. None of the pulmonary function parameters (total lung capacity, vital capacity, residual volume, FEV_1, and airway resistance) has any effects on EBC volume. Only ventilation plays an important role, where larger total respired volumes result in greater condensate yield [177]. This is why the collection should always be performed simultaneously with a measurement of the flow rate of respiration. There exist different types of condensation devices, both commercial and homemade. House built EBC collection devices are generally constructed using an externally cooled tubing made of various materials such as glass, polypropylene Teflon, or aluminum,

plunging in a glass double wall cooled by circulating icy water or liquid nitrogen using a pump. The distal end of the tubing contains either a collection tube or a container where droplets eventually drop below by gravity. Increasing number of studies use more frequently commercial devices to collect EBC because the latter have potentially better results in reproducibility of data [179]. These devices include R-Tube (Respiratory Research, USA), EcoScreen (FILT Lungen-& Thorax Diagnostik GmbH, Germany), TURBO-DECCS (Medivac, Italy), and ANACON (Biostec, Valencia, Spain). TURBO-DECCS is still the only device among those listed above whose the literature is not well documented, making it difficult to document its performances. Nevertheless, it has the advantage to have disposable connectors for sampling, which facilitate cleaning and the proper sterilization of the device between uses. The collection tube is the most critical and important part of the device; its surface area correlates with the amount of EBC collected. Its temperature during collection also plays an important role, especially for thermally labile compounds. The person must ensure a complete seal around the mouthpiece with his or her mouth, which must be kept dry by periodically swallowing excess saliva. The mouth should be rinsed thoroughly before the maneuver, and wiped every 5 minutes during the test. To avoid contamination, subjects are instructed to wash their hands prior to the collection of the EBC.

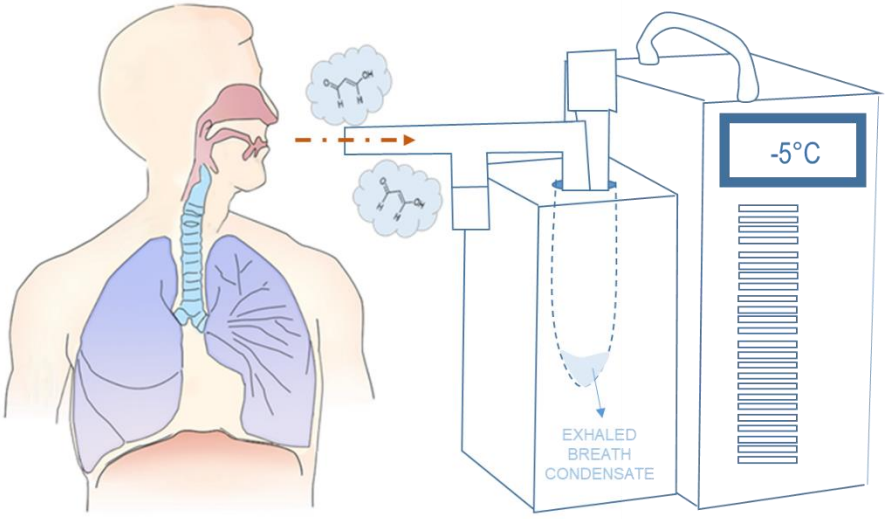


Figure 14. *Collection of EBC*

c. Considerations in condensate collection

There are important limitations to the use of EBC that should be noted. The limitations that influence the amount and quality of condensate collected include: duration and temperature at which the specimen is collected; the presence of contaminants such as saliva and dilution

factor of the respiratory droplets. EBC is collected from a large portion of the airway so the fluid collected may be derived from the large airway when it is supposed to be used to analyze only alveolar wall fluid [180]. It is assumed that the airway surface liquid is aerosolized during turbulent airflow, so that the content of the condensate reflects the composition of the airway surface liquid, although large molecules may not aerosolize as well as small soluble molecules [181]. Jackson et al., [182] demonstrated that EBC was extensively sampled from the alveolar wall fluid by observation of the surfactant concentration in their sample.

EBC is collected through the mouth, and the final sample may be affected by oral components (e.g., saliva and oral flora organisms). However, the extent of this contamination in EBC remains controversial. Currently, it is possible to identify salivary contamination by colorimetric detection of alpha-amylase activity [183].

The composition of exhaled air is influenced by the source of the air in the lungs. In general, a 50% increase in CO_2 concentration distinguishes bronchial air from alveolar air that contains the metabolites of interest. However, when collecting EBC, it is impossible to strictly discriminate exhaled air due to the turbulent motion during expiration that strongly influences its composition and mixes the different origins.

Another important element is the dilution factor of the respiratory droplets, which is not yet established. It depends on the efficiency of the collection system (coatings, materials used in the device...) and determines the biomarker recovery rate. Different dilution correction factors have been studied (e.g., urea, conductivity or total cations) to allow a better standardization of the EBC with a very wide range of reported physiological dilution rates [184,185].

Dilution factors may also differ between markers because they depend on the characteristics of each molecule (water solubility, hydrophilicity, volatility), which in turn may vary with the underlying diseases. The measurement in EBC stems from the idea that these biomarkers of interest present in airway lining fluid droplets would become totally aerosolized during exhalation [181]. There is no reason for believing that droplets would be formed in a constant fashion in the water vapor [186,187]. The droplet formation depends on the velocity of the flow of exhaled air and the surface tension (higher velocity/lower surface tension). The number of particles in exhaled air is very low, ranging from 0.1-4 particles/cm^3 [188]. According to the polarity of the biomarker this may tend to remain associated with surfactant in the lungs it would explaining why certain biomarkers will be present at an extraordinarily low concentration or be missing in the condensate [186,189]. In addition, the relative value of contribution of different parts of the airway to each biomarker is unknown.

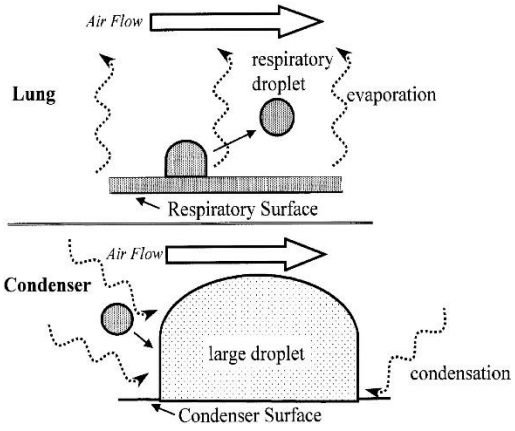


Figure 15. *Collection of respiratory droplets through condenser [185]. Variable quantities of droplets are released from the surfaces of the airways in the form of water vapor according to the polarity. In the second picture, the respiratory droplets reach the condenser, they deposit on its cold walls.*

d. Standardization of EBC sampling and storage

Before the EBC can be used for clinical use, it must demonstrate proper reliability. Currently none of the biomarkers analyzed in the EBC have been sufficiently validated for this purpose. With only a relatively small number of researchers studying each biomarker, the process of developing standardized methodologies for each individual biomarker is very slow. In addition, standardization of EBC sampling for widespread clinical use requires an international collaborative effort. In 2005, the American Thoracic Society (ATS)/European Respiratory Society (ERS) working group based on the consensus of the expert group published the first recommendations for collection and EBC analysis [180]. They provided guidelines on areas of uncertainty pending the implementation of standardized protocols, which would allow a more accurate and reproducible estimation of potentially measurable biomarker levels in EBC [179]. These recommendations summarized from articles can be listed in Table 5.

Table 5. *Recommendations from American Thoracic Society (ATS)/European Respiratory Society (ERS) for collection and EBC analysis*

Standardization issue	***Observations***	***Recommendation***
Sampling		
Device	Multiple collection devices are available (TURBO-DECCS, EcoScreen, R-tube…) and individual investigators decide upon device as well as protocol in terms of specimen collection.	Collection methods have been standardized, otherwise it will be difficult to reliably compare information between different investigations. There is no ideal device for collecting EBC for all biomarkers, since the concentrations of some biomarkers depend on properties of condenser coatings
Condenser	Collection systems have different coating materials, e.g., Teflon, polypropylene, glass, silicone or aluminum.	Surfaces contacting the EBC should be inert or must be standardized for each compounds of interest. Different biomarkers may have different reactivity with surface materials.
Temperature	EBC collection devices work at different cooling temperatures ranging from zero to below −20°C. Temperature at which EBC is performed has an impact on collection volumes	Investigators should report routinely collection temperature and keep the same sampling temperature in studies that require repeated sampling.
Duration of collection	Most studies report 1 ml to 3 ml of collected sample with collection times ranging from 5 minutes to 1 hour.	At least 10 minutes of collection time is enough for most mediators. In all cases this time should be routinely reported.
Contamination	Contamination of EBC specimens is mostly from the nasal airflow, oral and retropharyngeal portions of the airway. Saliva is the significant source for oral contamination.	Efforts to prevent salivary contamination should still be made using a saliva trap. Monitoring by amylase measurements are still controversial. Collections should occur during tidal breathing using a nose-clip.
Dilution	Dilution can come from multiple sources, including excess moisture in airway vapors.	Dilution factors have to be used in order to normalize concentrations of biomarkers collected in EBC when non-volatiles compounds are searched. (e.g., sodium, chloride, urea, protein concentration, conductance)
Storage		
temperature	EBC contains unstable volatiles	Immediately after collection, certain substances (e.g., pH, H_2O_2) have to be measured without

		freezing or storing EBC. For other substances, EBC should be stored in an inert material and be immediately frozen using dry ice or at −80 °C until analysis.
Stability		The effects of storage time and storage conditions on the stability of different components need to be carefully evaluated for each substance

e. Exhaled breath condensate and chronic obstructive pulmonary disease

We have reviewed a large body of data on concentrations in biomarkers exhaled in EBC. Despite some outstanding questions about the reliability of the present studies (*This issue will be addressed later in the part 2*), they demonstrate a new insight into the detection of COPD. They reveal interesting identifiable patterns of change in breath composition of biomarkers such as 8-isoprostane, 8-OHdG and MDA, between COPD and controls (Table 6, Table 7 and Table 8) and even within the different COPD severity grades [190-195]. These changes in biomarker profiles depend on the progression of the current disease and the inflammatory processes underway during the exacerbation. In view of these results, EBC have the potential to be useful as early-stage diagnostic and a prognostic tool and could help guide treatment. Clinicians may be able to monitor the disease with a simple, non-invasive sampling that can easily be repeated, allowing for frequent reassessment of the patient's condition.

Table 6. *Reported concentrations of 8-isoprostane in EBC of COPD patients versus controls*

Study	Year	Control				COPD				
		N	Age mean ± SD or range (years)	Gender (M/F)	Values (pg/ml)	N	Age mean ± SD or range (years)	Gender (M/F)	Values (pg/ml)	FEV_1 (%) mean ± SD
Antczak [196]	2011	13	57 ± 19	10/3	**31.3 ± 4.27** [d]	16	64 ± 12	13/3	**60.9 ± 3.84** [d]	62 ±2
Ashmawi [190]	2018	20	41.8 ± 7.1	16/4	**6.3 ± 1.5** [b]	80	51.7 ± 9.9	62/18	**24.9 ± 4.4** [b]	54.8± 18.3
Biernacki [197]	2003	12	56 ± 4	8/4	**6.2 ± 0.4** [d]	21	66 ± 8	11/10	**15.8 ± 1.1** [d]	51±13
Brindicci [198]	2009	10	57.2 ± 6	6/4	**11.5 ± 0.9** [d]	10	62.4 ± 3	6/4	**43.7 ± 2.8** [d]	62.9 ± 5.6
Carpagnano [191]	2004	15	42	8/7	**4.5±0.8** [b]	5	53±6	3/2	**18.9±3.6** [b]	60.9±2.6
Fritscher [199]	2012	22	37.5 ± 10.4	8/14	**0.9 (0.2–1.7)** [c]	20	65.8 ± 7.3	8/12	**0.5 (0.2–1.4)** [c]	48± 15.3
Inonu [200]	2012	26	61.2 ± 6	26/0	**41.3 ± 26** [b]	25	63.3 ± 7	25/0	**44.8 ± 40.2** [b]	57.8±7.3
Kazmierczak [192]	2015	16	53.1 ±7.31	4/12	**9.3 ± 7.43** [b]	20	65.05 ± 8	10/10	**18.2 ± 20.65** [b]	57.78±10.12
Ko [193]	2006	18	73 ± 5	13/5	**6 (4.2-9.7)** [a]	32	72 ± 9	28/4	**14.2 (9.5-26.4)** [a]	51.5±22.4
Kostikas [201]	2002	10	34±8	7/3	**20±7** [b]	20	54±8	14/6	**60±44** [b]	59 ±14
Makris [194]	2007	12	61	3/9	**5.6±0.7** [d]	18	64	6/12	**18.1±2** [d]	47±5
Montushi [202]	2000	10	63±4	5/5	**10.8 ± 0.8** [b]	25	66±3	12/13	**39.9 ± 3.1** [b]	54.1± 3.1
Pirozzi [203]	2015	9	66.8-5.6	4/5	**12.3±9.7** [b]	11	70.7±4.7	8/3	**5±5.6** [b]	39.1± 16.6
Romero [204]	2005	14	62.86±2.96	12/2	**3**	14	68.14±1.88	14/4	**18(16-26)** [a]	-
Santini [205]	2016	12	56	9/3	**8 (6-8.8)** [c]	48	69	36/12	**17.8 (8.8-31.2)** [c]	58.0± 2.4

COPD: Chronic obstructive pulmonary disease; M: male F: female N: number of people; SD: Standard deviation. [a] values are presented as medians (25th-75th percentiles); [b] Values are presented as mean (±SD); [c] values are presented as median (range); [d] values are presented as mean (±SEM).

Table 7. *Reported concentrations of 8-OHdG in EBC of COPD patients versus controls*

Study	Year	Control				COPD				
		N	Age mean ± SD or range (years)	Gender (M/F)	Values (pg/ml)	N	Age mean +/- SD or range (years)	Gender (M/F)	Values (pg/ml)	FEV_1 (%) mean ± SD
Fireman [206]	2019	40	57.2±14.4	21/19	**3**	58	68.3±8.6	46/12	**36**	

COPD: Chronic obstructive pulmonary disease; M: male F: female N: number of people; SD: Standard deviation.

Table 8. *Reported concentrations of MDA in EBC in of COPD patients versus controls*

Study	Year	Control				COPD				
		N	Age mean ± SD or range (years)	Gender (M/F)	Values (pg/ml)	N	Age mean +/- SD or range (years)	Gender (M/F)	Values (pg/ml)	FEV_1 (%) mean ± SD or range
Antus [195]	2014	20	61.8±1.6	10/10	**5268 ± 368** [d]	21	63.1±1.8	13/8	**6925 ± 836** [d]	63.1±4.3
Bartoli [207]	2011	14	40.4±13.1	6/8	**1124 (288-1802)** [a]	73	71.1± 7.6	60/13	**2666 (576–9080)** [a]	49.8± 17.7
Corradi [208]	2003	20	55±6	17/3	**872 ± 130** [d]	20	63.3±14.6	18/2	**4122 ± 173** [d]	52.1 ±20.1
Corradi [209]	2004	9	32 (25-50)	1/8	**706 (663-764)** [c]	11	74 (34–83)	10/1	**1729 (850–2349)** [c]	66.5 (45–82)
Inonu [200]	2012	26	60 ± 8	26/0	**5765±1441 ****	25	63.3±7	25/0	**5765±721 ****	57.8 ± 7.3
Lee [210]	2015	53	62.1 ± 8.0	53/0	**334±192** [b]	37	65.1 ± 7.4	37/0	**478±245** [b]	62.7 ± 7.4
Warzęchowska [211]	2017	19	23 (21–24)	11/8	**1422 (1292-1584)** [c]	19	65 (61–69)	11/8	**1366 (1175-1443)** [c]	61 (50–86

COPD: Chronic obstructive pulmonary disease; M: male F: female N: number of people; SD: Standard deviation. [a]values are presented as medians (25th-75th percentiles); [b] Values are presented as mean (±SD); [c] values are presented as median (range); [d] values are presented as mean (±SEM).

3. Oxidative potential in exhaled air

There is a wide panel of chemical/biochemical colorimetric assays routinely used to direct and quickly quantitatively detect specific volatile analytes samples in exhaled air. These essays include electronic noise [212], electrochemical [213] or surface-enhanced Raman scattering (SERS) [214]. For the measure of oxidative stress species in air (mainly formed of H_2O_2) current colorimetric essays are not sensitive enough because these latter are present in very low concentration. Our team developed a new, cheap and versatile device using sensitive photonic detection system based on multiscattering-enhanced absorbance strategy to measure oxidative species in exhaled air or commonly called oxidative potential. This device named OPEA analyzer, measures OPEA, reflecting the state of balance between oxidizing and reducing molecules with respect to the Fe(III) / Fe(II) couple, expressed in H_2O_2 concentration equivalent (mol H_2O_2/L_{air}) . This device will be discussed in more detail in the following chapters.

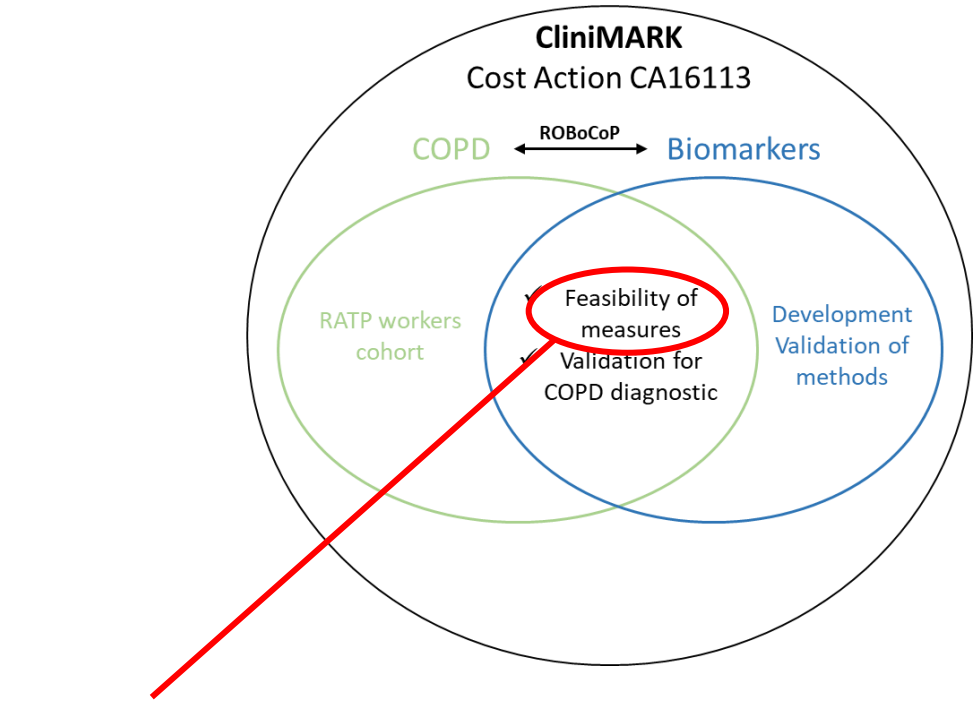
FRAMEWORK OF THE THESIS

Inhalation of particulate matter, via an immune mechanism, tends to induce inflammation and endogenous ROS production. ROS overproduction, in turn, lead to oxidative stress, inflammation and ultimately increase the risk of COPD. The diagnosis and assessment of COPD currently relies on respiratory functional exploration test using spirometry. However, this technique does not allow early diagnosis nor early detection of exacerbation episodes.

As a complementary approach to spirometry, several internationally research teams have investigated the usefulness of oxidative stress biomarkers to detection and monitor COPD evolution and severity with some promising results. However, in the case of EBC these methods lack of adequate validation and standardization. These current reliabilities issues hinder the possible translation of oxidative stress biomarkers toward clinical practice.

Over the past ten years, our laboratory has developed an expertise in EBC collection and in the development of methods for the analysis of oxidative stress biomarkers. In April 2017, the laboratory joined the European consortium CliniMARK (EU COST Action CA16113) with two main strategies: 1) Accelerate the development and validation of new methods for the analysis of oxidative stress biomarkers, based on mass detection techniques instead of current use of ELISA, by respecting the rules of good practice in this field, at an European level. 2) Propose, in the foreseeable future, the use of the most promising oxidative stress biomarkers for the diagnosis or accompanying the diagnosis of COPD.

In an effort to bring this complex and challenging project to completion, our laboratory partnered with RATP (Autonomous Parisian Transportation Administration) and created a common project: the Respiratory disease Occupational Biomonitoring Collaborative Project (ROBoCoP) supported by the Swiss National Science Foundation. The RATP's underground rail enclosures are identified as a particularly particulate polluted area. Exposures to such high concentrations of particulate matter over a prolonged period of time may significantly increase the risk for RATP workers of developing COPD. When workers develop COPD, one third have to stop working definitively due to their respiratory problems. An early diagnosis before the onset of COPD could represent a real hope for these workers. Oxidative stress biomarkers have the potential to be used for reliable non-invasive screening COPD provided that their feasibility can be previously assessed in the field. These issues of feasibility and usefulness for screening COPD will be addressed in two field studies, respectively, in collaboration with RATP.



My PhD work entitled *"Novel analytical methods for the non-invasive measurement of oxidative stress in exhaled air: application to workers in underground railway enclosures"* is embedded in this project from the development and validation of analytical methods to the assessment of the feasibility of using biomarkers in the field.

The main objectives of the thesis are:

Objective 1: Standardization of biomarkers detection techniques in exhaled air

1.1 *Improve existing analytical methods in exhaled breath condensate.*

This work was focused on the detection techniques of oxidative stress biomarkers that can be measured non-invasively such as in exhaled breath and previously identified in literature review as associated with COPD diagnosis. Our approach focuses on the quantification of a panel of three oxidative stress biomarkers in EBC using liquid chromatography coupled with mass spectrometry detection (LC-MS/MS): 8-OHdG, 8-isoprostane and MDA. To be useful, those techniques must be robust and highly sensitive. Providing that EBC is a strongly diluted sample, the main challenge consisted of reaching low limit-of-quantification as well as robust sample pre-concentration step. All factors including potential interferents that can influence concentrations in this matrix must will attempt to be resolved before a potential field application.

1.2 *Optimize and characterize the determination of oxidative potential in exhaled air.* In a complementary approach, we develop a portable photonic system able to determine the oxidative potential mainly constituted of H_2O_2 in the exhaled air (OPEA) via direct analysis. The detection principle relies on multi-scattering enhanced absorbance strategy that enables fast and sensitive determination of the homeostatic redox balance if lining fluid droplets are contained in the exhaled breath aerosol. OPEA analyzer system were optimized and tested to achieve robust and sensitive measurements adapted for further epidemiological studies. The implementation of a capnometer (CO_2 detector) in the current oxidative potential measurement system were studied in particular. The capnometer allows to fractionate the exhaled air in order to distinguish between the alveolar and bronchial air collection. The measurement of oxidative potential on fractionated samples will enable to observe the relative magnitude of the inflammation in the bronchi and alveoli, which may notably differ among COPD patients.

Objective 2: Validate the feasibility of selected oxidative stress biomarkers in the perspective of their application in large scale field study

The aim for this field study is to identify if the selected oxidative stress biomarkers in EBC and exhaled air can ideally provide reliable information on the human lungs depending on the type of exposition in Paris metro stations. This were undertaken through:

- Definition of the feasibility framework for the measurement of selected biomarkers in PM-exposed workers in comparison with some other biomarkers in EBC (such as markers of nitrosative stress: NO_2^-, NO_3^- and formate ion; nanoparticle tracking analysis; exposure biomarkers metals)
- Estimation of exposure through the use of determinants and integrative exposure measures;
- Understanding of relationships between parameters characterizing exposure and biomarkers response;
- Selection of the best suitable biomarkers than could be measured on the future study.

Objective 3. Comparison of the results obtained with the values found in the literature

Finally, these results will be compared with reference values. Baseline values for these oxidative stress biomarkers are still not known. For this reason, we initiated a literature review and meta-analysis to obtain the baseline values of several oxidative stress biomarkers, 8-OHdG, 8-isoprostane, MDA in EBC in a healthy non-smoking adult population according to sex and age, as well as body mass index. Literature searches were conducted since journal inception and up to April 2019 (8-OHdG/ 8-isoprostane) or October 2020 (MDA) in the following bibliographic electronic databases: The Cochrane Central Register of controlled Trials, EMBASE, PubMed, and Web of Science.

In summary, this research thesis project encompasses three main parts:

- Standardization of techniques for detecting oxidative stress biomarkers in exhaled air;
- Application of these techniques to an occupational field study at Paris underground;
- Comparison of the results obtained with the values found in the literature.

STANDARDIZATION OF BIOMARKERS DETECTION TECHNIQUES IN EXHALED AIR

Whether we expect to guide important clinical decisions, have personalized treatments for COPD based on the results of chemical quantitative analysis, it is important to ensure our oxidative stress biomarkers are reliable and provide high-quality results. Validation is one of the essential conditions to ensure that the biomarkers will be able to provide it. Validation involves the comprehensive evaluation of performance characteristics during method development, such as, precision, accuracy, detection limit and robustness, and the search for potential interferents.

In exhaled air media, biomarkers are often detected at concentrations in the nanomolar range. These levels are far too low to be reliably interpreted by simple colorimetric methods such as enzyme-linked immunosorbent assay (ELISA). These biomarkers are also prone to significant effect of potential interferents or a lack of reproducibility because values are often close to the detection limit. Over the last few decades, new analytical techniques have been developed and optimized to achieve the necessary sensitivity/specificity for the analysis of these biomarkers. These new methods have ranged from gas or liquid chromatographic techniques based on mass spectrometric detection, to ultra-sensitive technologies based on photonic systems for direct measurement of ROS levels in exhaled air in a rapid and non-invasive manner (OPEA) [215].

I. Improvement of existing analytical methods in exhaled breath condensate

So far, none of these gas or liquid chromatographic techniques based on mass spectrometric detection methods has yet demonstrated real reliability in EBC. The used methods are either unreliable, which is the case for the current MDA measure, or very inconsistent, for 8-OHdg and 8-isoprostane.

In this study, I propose an alternative robust protocol to assess the presence of 8-OHdG/8-isoprostane simultaneous and MDA in EBC using High-Performance Liquid Chromatography – tandem Mass Spectrometry (LC-MS/MS) method to separate and selectively detect the biomarkers. The methods we intend to develop requires no manual tedious purification process unlike some previous studies and use equipment that is now commonly available and routinely operated in most analytical laboratories. This choice is based on a willingness to apply such methodology in the context of occupational epidemiology studies, where a high-through put and cost-effective analysis have to be preferred.

The main challenge has consisted of reaching low limit-of-detection that corresponds to the levels observed for healthy non-smoking population while paying attention to possible interferences.

The second challenge has consisted of detecting simultaneously 8-OHdG, 8-isoprostane and MDA derivatized with 2,4-Dinitrophenylhydrazine (DNPH), in the same analytical method since EBC is available in small quantities but significant issues have been quickly encountered. These issues are: I). the derivatization agent was interfering with 8-OHdG and 8-Isoprostane decreasing the sensitivity of our analysis II). The levels of 8-OHdG and 8-isoprostane present in the EBC were in the order of magnitude well below that of MDA concentration and a concentration step inexorably increased the concentration of DNPH to a level that quenched the 8-OHdG and 8-isoprostane signal. Finally, this first approach was discarded and two analytical methods have been developed and published in two separate experimental studies.

The methods and results are presented in the continuation of the chapter. These results aim to support the adoption of consensus criteria with respect to assay performance and thus minimize inter-laboratory variance in the future.

1. Development of method for MDA

a. Context

MDA is one of the most commonly used biomarkers of OS. Its concentration can be determined by several methods, including a derivatization step with DNPH analysis by LC-MS [216]. This derivatization contributes to an improved chromatographic separation, mass spectrometer (MS) ionization and MS/MS fragmentation detectability [217,218].

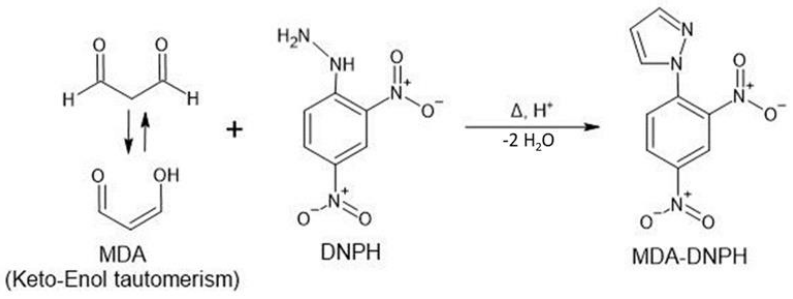


Figure 16. *MDA derivatization process with DNPH yielding 1-(2,4-dinitrophenyl) pyrazole (MDA-DNPH). At lower pH MDA appears in equilibrium between its protonated enol (α-β-unsaturated carbonyl) aldehyde form and the dialdehyde form*

This method presents a thorough work on the improvement of MDA quantification in EBC including method development and validation with special emphasis on uncertainty characterization. Uncertainty is a term to describe the dispersion of the values around a target value. This dispersion is greater for values close to the detection limit and has the potential to mark the signal in the region comprised between LOD and LOQ, which is precisely the domain where most of our values of MDA are found.

Uncertainty may arise from many sources, including contaminants in solvents, reagents, glassware and other sample processing materials. In the context of the MDA, the contamination of DNPH reagent could be one of the main sources of uncertainty. DNPH could act as a passive cartridge capturing the MDA prevalent in the environment and thus contribute to an overestimation of the actual concentrations of MDA that can be found in our samples.

This method attends to be the first attempt to identify and characterize potential impact of all uncertainties sources, with the analysis of MDA in EBC, and statistically demonstrate that method could still perform sufficiently well to be used in clinical researches.

This work was described in the scientific manuscript entitled "Method Validation and Characterization of the Associated Uncertainty for Malondialdehyde Quantification in Exhaled Breath Condensate" **published** as original article in the Special Issue "Biomarkers of Oxidative Stress in Acute and Chronic Diseases" of the journal of **Antioxidants.**

b. Materials and Methods

Reagents- chemicals

MDA-salt (MDA tetrabutyl ammonium salt) (96%, neat) was obtained from Sigma-Aldrich (St. Louis, MO, USA). MDA-d2 (1,1,3,3-tetraethoxypropane-d2, stock solution: 98%) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). DNPH reagent (2,4-Dinitrophenylhydrazine) in a solid form containing 25% water, was obtained from Carlo Erba Reagents (Chaussée du Vexin, Val de Reuil, France). HPLC grade methanol (≥ 99.9%) was obtained from Merck (Buchs, Switzerland). LC-MS grade solvents, methanol (≥ 99.95%) and acetonitrile (≥ 99.9%) were obtained from Carlo Erba Reagents (Chaussée du Vexin, Val de Reuil, France). LC-MS grade acetic acid was obtained from Honeywell (Seelze, Germany). High purity water was produced in our laboratory with a Milli-Q Advantage water purification system (18.2 MΩ.cm at 25°C, < 3 ppb total organic carbon; Merck, Schaffhouse, Switzerland).

Preparation of standards and procedural blanks

A stock solution of MDA at 5.4 µg/mL was prepared by diluting a weighted mass of MDA tetrabutyl ammonium salt (7 mg) in 3 mL of MeOH and further diluting it by a factor of 100 with Milli-Q water. The MDA stock solution was stored at -80 °C during 8 months. To overcome unwanted variations during derivatization and analysis, MDA-d2 was used as internal standard (IS). The MDA-d2 stock solution was prepared by acidic hydrolysis of the 1,1,3,3-tetraethoxypropane-d2 standard according to a previously published protocol [219]. Briefly, 1,1,3,3-tetraethoxypropane-d2 (50 mg) were poured into 28 mL of 0.02M HCl and left for 2 hours at room temperature. The resulting MDA-d2 stock solution at 7.9 mM in 0.02M HCl was then stored at 4°C. These different stock solutions were diluted in Milli-Q water to prepare daily working solutions of MDA (1 and 20 ng/mL) and IS (283.5 ng/mL). Calibration standards were obtained by diluting the working solutions to get final MDA concentrations of 74, 148, 370, 740, 1110, 1480 and 2220 pg/mL, with a constant IS concentration of 15 ng/mL. The concentration of the internal standard was selected to be of comparable signal intensity observed for the MDA calibration standards. The criteria for linearity were assessed by means of the coefficient of determination (R^2), fixed at $R^2 > 0.99$. Procedural blanks correspond to Milli-Q water, with IS at concentration of 15 ng/mL.

EBC samples and quality control (QC)

EBC samples used for validation and quality control (QC) were collected from 13 healthy non-smoking voluntary adult participants. The sample included 9 women and 4 men. For EBC collection, we used the commercially available breath condenser (Turbo Deccs, Medivac, Parma, Italy). All collected samples were pooled, aliquoted in plastic tubes (1 mL) and stored at −80◦C until analysis. These samples were used during the method development and validation, particularly for investigating the matrix effect and the limit of detection (LOD) for MDA, as well as QC during sample analysis. An average concentration of these pooled samples was calculated over twelve independent measurements to determine a baseline concentration. Then, they were spiked with known MDA concentrations and used for QC. QC were prepared at final concentrations of 211, 349, 642, 1180, 2258 pg/mL with IS at 15 ng/mL. Each analysis sequence for validation included a seven-point calibration curve in duplicate, five EBC QC samples in quintuplicate, two non-spiked EBC and six procedural blanks (Figure 17).

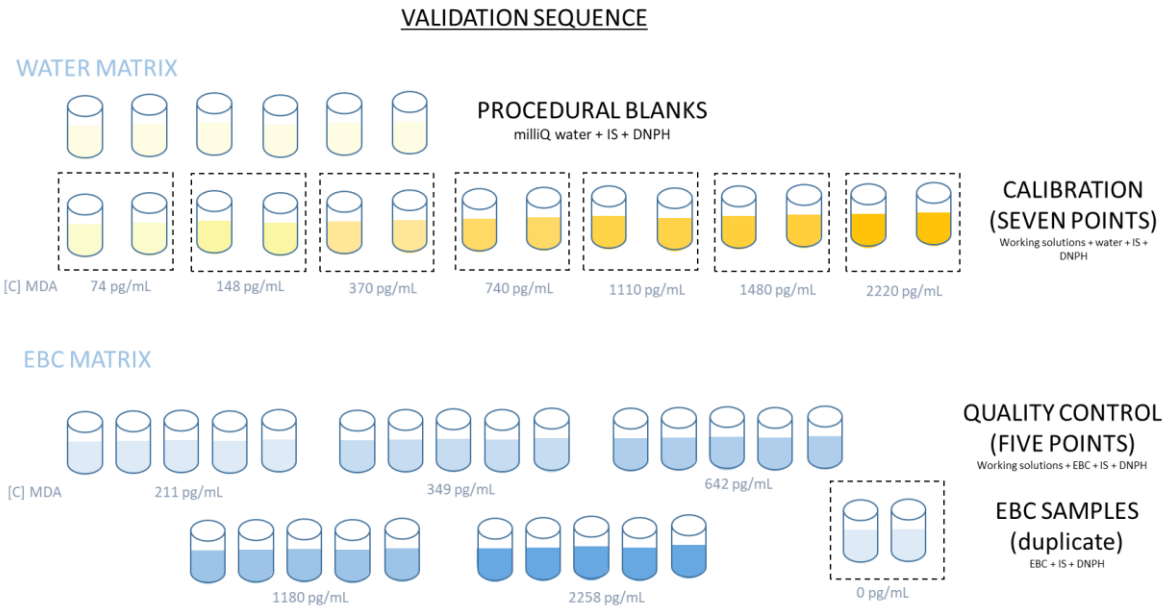


Figure 17. *Validation sequence in three different days*

For assessing the suitability of the validated method, we used 164 EBC samples collected in an occupational field pilot study. (*Refer part 3*) This study included nine non-smoking healthy adults of both sexes working in the underground subway in Paris. Three workers from three occupational groups were included. EBCs were collected twice daily (before and after the working shift) during ten days following the latest recommendations of the American Thoracic

Society and the European Respiratory Society Task Force [220]. Food and drinks consumed within 3 hours before EBC collection were recorded in a standardized form. None of the participants declared drinking coffee within the hour before EBC collection. The exhaled air was condensed at -10 °C during calm oral respiratory ventilation for 2x10 minutes, using the Turbo Deccs. EBC (2-3 mL) was collected and aliquoted immediately away from the sampling area on a clean table. Collected EBC aliquots were frozen at -20 °C, transported, and stored at -80 °C until analysis. Nine analytical sequences (one for each volunteer) including one calibration curve (seven levels), the EBC samples, six procedural blanks and two QC controls (low: 211 pg/mL and high: 2258 pg/mL) were analyzed.

MDA derivatization with DNPH

The DNPH derivatizing solution was prepared at a concentration of 396 µg/mL in a H2O:ACN:acetic acid mixture (6:3.8:0.2 v/v, pH≈ 3.2) and stored dark at room temperature. Water was added gradually at the last stage to avoid precipitation of DNPH due to its low solubility in aqueous solution. The MDA derivative was prepared by incubating 135 µL of the sample (including 10 µL of IS) with 50 µL DNPH 396 µg/mL for 2h at 50 °C. We found that this condition was sufficient for a complete reaction between the MDA and DNPH present in excess (Figure 18). The resulting mixture was immediately analyzed by HPLC-MS/MS after cooling to reduce possible interferences originating from additional sample treatment.

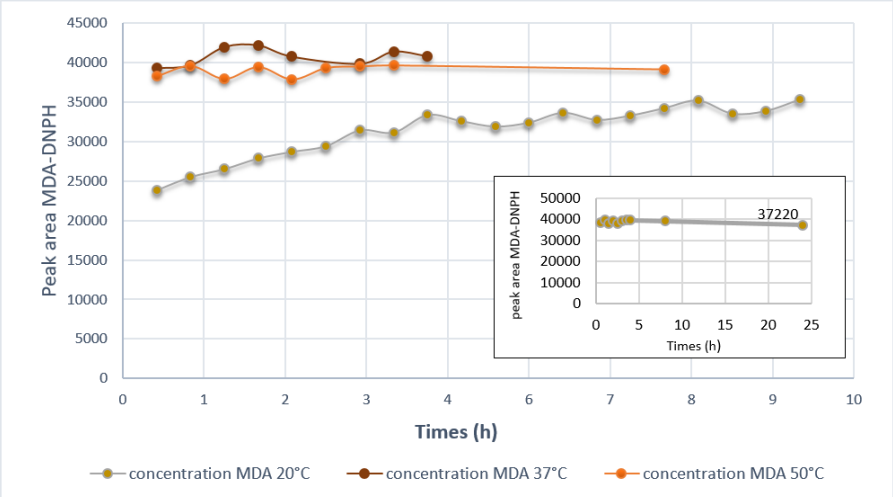


Figure 18. *Effect of the reaction temperature on the MDA-DNPH formation as a function of time. The small box represents the variation of the MDA concentration at 20°C over a period of 24 hours*

HPLC – MS/MS analyses

The target analytes were analyzed with an ultra-high pressure liquid chromatography (LC) system (Dionex Ultimate 3000) coupled with a Triple-Stage Quadrupole MS (TSQ Quantiva Thermo Scientific-Reinach, Switzerland). A C18 column (Zorbax Eclipse Plus 2.1 x 100 mm, 1.8 µm, Agilent, Morges, Switzerland) at 30 °C was used for separation. The injection volume was 20 µl and the solvent gradient (flow rate of 0.25 mL/min) combined eluent A (H_2O with 0.1% acetic acid) and eluent B (MeOH/ACN 7:3 with 0.1% acetic acid). The following program was used: 100% A at 0 min, decreasing to 45% at 1.1 min, then to 35% A at 5 min, then to 10% A at 5.5 min until 7.5 min and increasing to 100% A at 8 min until 14 min.

The detection of MDA-DNPH was performed through a heated electrospray ionization (ESI) source operated in positive ion mode with the following parameters: spray voltage, 3700 V; ion transfer tube temperature, 390 °C; and vaporizer temperature, 350 °C. For MDA-DNPH, the transition m/z 235→159 was used for quantification whereas the two other transitions m/z 235→143 and m/z 235→189 were used for confirmation; for MDA-d2-DNPH, the quantification transition was m/z 237 → 161. Chromatography Data System software (version 7.2.10, Thermo Scientific Dionex Chromeleon 7) was used for data acquisition and processing.

Method validation and estimation of its expanded uncertainty

The validation of the optimized method was carried out on three different days by considering linearity, limit of detection (LOD) and quantification (LOQ), intra-day and inter-day precisions, recovery as a measure of accuracy, storage stability and matrix effects as described in FDA/ICH guidelines [221].

Limit of Detection (LOD) was calculated as 3 x intercept error on the slope of the calibration curve for samples. It was calculated over 12 days in accordance with [222].

Limit of quantitation (LOQ) is the smallest concentration at which the analyte can be quantified reliably, with an observed bias and imprecision smaller than an acceptable maximal deviation set at 20%. The LOQ was calculated by multiplying the LOD with a factor of 3.

Precision

The method precision was determined by analyzing in quintuplicate, five levels of EBC QC, and five different EBC QC (corresponding to the intra-day precision). This measurement was repeated three non-consecutive days over a two-week period (corresponding to the inter-day

precision), as described in the FDA/ICH guidelines [221].The precision were examined as the intra-day precision (repeatability) and the inter-day precision (intermediate precision) following an ANOVA-based variance decomposition and expressed as relative standard deviation (% RSD) values (equation (1)).

$$\text{Method precision} = \sqrt{\text{Sr}^2 + \text{Sb}^2} \quad (1)$$

Repeatability (Sr) (% RSD) was calculated as square root of the repeatability variance corresponding to the sum of daily variances divided by the number of days (equation (2)).

where Si^2 is the daily variance, n the number of days.

$$Sr^2 = \frac{\sum_{i=1}^{n} Si^2}{n} \quad (2)$$

The intermediate precision (Sb) (% RSD) was calculated as the square root of the inter-daily variance corresponding as the mean of the daily variances minus the repeatability variance divided by the number of replicates (equation (3)).

$$\text{Sb}^2 = s(\overline{zi})^2 - \frac{Sr^2}{r} \quad (3)$$

where, $s(\overline{zi})^2$ is the variance of daily averages, r the number of replicates.

Matrix effects

The matrix effect was evaluated by comparing the slopes of the calibration curves obtained in water and in EBC, using QC samples across different analytical batches. An unpaired t-test was used for statistical comparison. A p value of 0.05 was used as the cut-off for significance.

Recovery rate

The recovery rate was calculated as the mean of quintuplicate QC solutions obtained on the validation process divided by the expected concentration values for each level (n=5). The recovery rate was expressed as percentage values (% RR) [221] (equation (4))

$$\textit{Recovery rate \%} = \frac{\text{practical concentration QC at level i}}{\text{Theoretical concentration QC at level i}} * 100\% \quad (4)$$

Accuracy

Accuracy was calculated as the relative back calculated concentrations (evaluated from the calibration curve), with respect to their targeted concentrations (expressed in percentage of variation from the targeted concentration) with an acceptance limits of bias set at 20% [223].

Storage

The stability of the MDA-DNPH derivative at room temperature (23 °C) was studied by reanalyzing samples kept in the auto-sampler after 24 or 48h. QC solutions and non-spiked EBC sample were also re-analyzed after 8 months storage at -80 °C.

Some of these validation parameters were used to estimate the expanded uncertainty of this analytical method [224]. We adopted a pragmatic approach to identify the main elements of uncertainty, using the overall method performance. We considered three parameters as main contributors to uncertainty: precision, recovery and purity of the MDA-salt. Precision represents all the effects covered by the intermediate precision study. It takes into account the daily variability of the calibration, including the different volumetric measuring devices (flasks and pipettes) used during the investigation. The recovery provides an indication of the accuracy of the concentration effectively found and consequently is subject to a degree of uncertainty. Finally, the MDA tetrabutyl ammonium salt used in this study is not 100% pure because it contains inorganic salts.

The general relationship between the combined standard uncertainty (u_c) of a given MDA concentration in EBC and the uncertainty of the independent parameters is defined by the following equation (5):

$$u_c\,(y(x1, x2, x3)) = \sqrt{\sum_{i=1,n} Ci^2 u(xi)^2} \qquad (5)$$

where y(x1, x2, x3) is the function of the 3 considered parameters: precision, recovery, purity, Ci is a sensitivity coefficient evaluated as ci=∂y/∂xi the partial differential of y with respect to each parameter. Ci describes how the value of y varies with changes in the parameters. u(xi) corresponding to the uncertainty related to each parameter is expressed as a standard deviation assuming Gaussian distribution of the parameters. The final expanded uncertainty is expressed by multiplying uc with a coverage factor of 2 (to have a β-expectation tolerance interval at 95%). An example of calculating the uncertainty at the LOQ can be found bellow:

Based on validation data, three major contributions to uncertainty and their relative uncertainties are listed in the table below:

Table 9. *Quantification of the uncertainty components at LOQ (209 pg/ml)*

Description	Value x	Standard uncertainty u(x)	Relative standard uncertainty u(x)/x	Comments
Bias/recovery	0.924	0.037[a]	0.040	Based on validation data (n=15)
Precision	1.0	0.11[b]	0.110	Based on validation data (n=15)
Purity	1.0	0.023[c]	0.023	Indication on the bottle
u_{total} (x)			0.11	
Coverage factor (95%)			2	
Expanded uncertainty			**0.23**	

[a]: In the validation procedure, a value of 92,4% was found with a deviation standard (s) of 13%. The standard uncertainty was calculated as : s /√n.

[b]: the standard uncertainty was calculated as the combination of repeatability and intermediate precision.

[c]: the standard uncertainty was calculated as 4% of impurity (given by the producer) divided by √3 (rectangular distribution).

Statistical analysis

In order to define the best calibration curve, we used the least square linear regression with an application of 1/x2 weighting as recommended by Gu et al., [225]. Coefficients of variation (CV) were calculated from the standard deviation and mean values. Basic calculations were performed with the built-in statistical functions in Microsoft Excel version 2016, whereas ANOVA, t-test, Tukey's test calculations were performed with the R software (R version 4.0.2.). For all the EBC samples with concentrations lower than the LOD, a value of LOD/2 was attributed for statistical analysis [226,227].

c. Results

LC-MS/MS analysis

LC separation and MS detection of MDA-DNPH in EBC was optimized to meet the highest sensitivity and repeatability. Due to the high concentration of DNPH reactive in the sample, the LC mobile phase gradient was optimized to guarantee a sufficient separation between the DNPH reactive and the MDA-DNPH analyte and to avoid signal suppression. The 6 min

washing period with eluent A at the end of the gradient program avoided a carry-over between different samples and protecting the analytical LC-MS system from solid deposition [228]. After derivatizing with DNPH, the MDA-d2-DNPH retention time was identical to the MDA-DNPH and only the m/z +2 mass of 237 was detected. The suitability of using MDA-d2 as IS for MDA quantification was thus confirmed.

Optimization of the DNPH derivatization

Optimization phases focused on the most complete formation of the desired hydrazine derivative. As protons play a significant role in the derivatization process (Figure 16), different acids (perchloric acid, formic acid or acetic acid) were tested on standard solutions as well as on EBC samples. Acetic acid had a significantly lower variation in blank values response compared with the other tested acids (one-way ANOVA followed by Tukey's test, $p < 0.05$; Figure 19). These variations were probably due to unwanted secondary reactions occurring at low pH and under oxidizing conditions, such as with perchloric acid. The final concentration of acetic acid was adjusted at 2% as we observed a lower procedural blank signal when increasing the concentration of water.

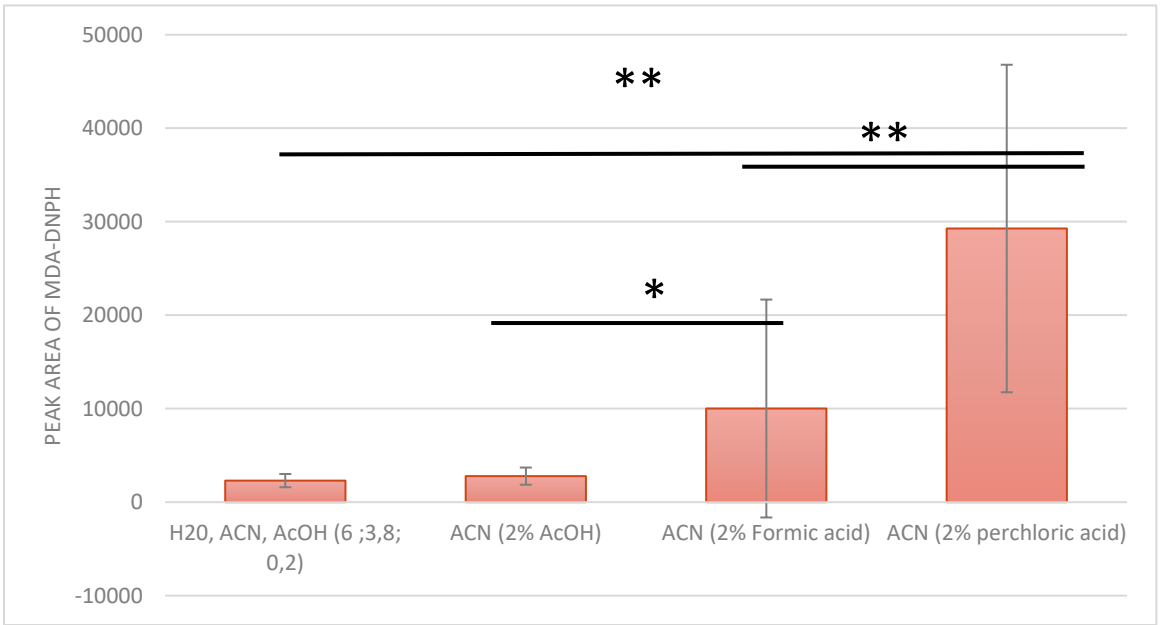
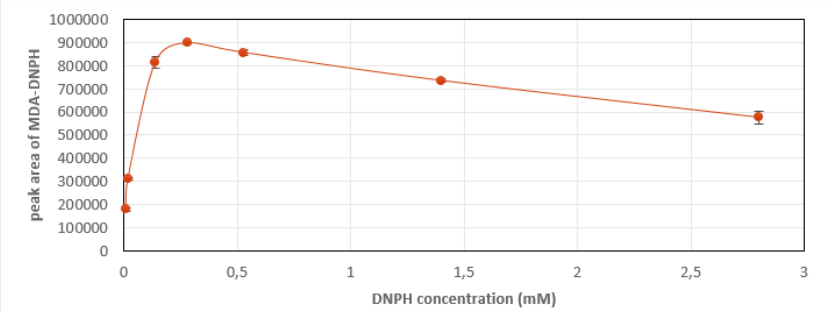
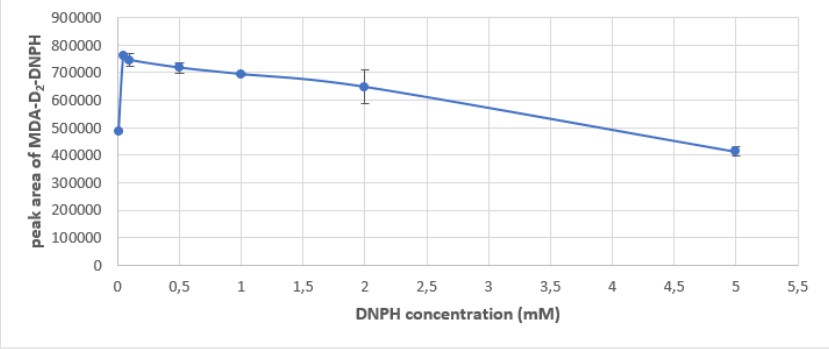


Figure 19. *Contribution of the acid type on the signal of MDA-DNPH in procedural blanks. The error bars correspond to the standard deviation of five measurements. Comparisons were made by one-way ANOVA followed by Tukey's test (*p< 0.05; **p< 0.005). AcOH: acetic acid; ACN: acetonitrile.*

To establish the optimal ratio of DNPH required to get an efficient derivatization of MDA in the sample, increasing DNPH concentrations in H_2O:ACN:acetic acid mixture (6:3.8:0.2 v/v) from 10 to 1980 µg/mL was applied to MDA standard solutions at 35 ng/mL. This latter concentration corresponds to the highest MDA concentration in EBC reported in previous studies [229-231]. An increase of the MDA-DNPH peak area was observed with increasing molar ratios. The largest peak area of the MDA-DNPH derivative was obtained at a molar ratio of DNPH:MDA of 1600, corresponding to a final DNPH concentration of 55 µg/mL (Figure 20a). This ratio is high due to the kinetics of reactions between DNPH and MDA with a first reaction instantaneously involving DNPH and one of an aldehyde function of MDA and a second slower reaction leading to the closing of the pyrazole cycle by H_2O elimination. In addition, DNPH could react not only with MDA but also with other aldehydes and ketones present in EBC samples. Thus, a large excess of DNPH was necessary to drive the reaction towards the MDA-derivative complex. For greater DNPH concentrations, the signal slowly decreased, suggestive of a signal suppression. In a parallel test with MDA-d2, a similar curve was observed as shown in Figure 20b, demonstrating a proper choice of ratio for DNPH.



(a)



(b)

Figure 20. *Effects of the added DNPH concentration expressed in mM on a) the peak area of MDA-DNPH b) the peak area of MDA-d2-DNPH for a standard solution at 35 ng/mL. The error bars correspond to the standard deviation of three measurements.*

The effect of temperature on the derivatization reaction was examined in the range of 20-50 °C. We originally selected the incubation temperature of 37 °C, as it corresponds to the physiological temperature and exhibited the highest HPLC-MSMS signals (Figure 18). However, we observed that in these conditions, the derivatization rate was difficult to keep constant, and thus selected incubation at 50 °C for 2h.

Figure 22 shows a typical liquid chromatogram for a procedural blank as well as the lowest standard level and an EBC sample using the optimized conditions. For the procedural blank, the signal at 7.35 min was identified as MDA-DNPH, as both the quantification ion (m/z 159) and confirmation ions (m/z 143 and m/z 187) presented the same retention time as the standard MDA. This indicated that a MDA source was present in the reactives used for this analysis. The contribution of this procedural blank to the signal of the lowest MDA concentration (74 pg/mL) was relatively large and variable, ranging from 45% to a maximum of 90%, depending on the day (SD: 15%, n=12). To decrease such interferences, we tried to purify the DNPH derivatization solution by using a liquid-liquid extraction following Mendoca et al., [232]. However, this approach was unsuccessful, attributed to the small polarity difference between DNPH and MDA-DNPH. Changing the type of glassware, tips and other crimp caps did not reduce this contamination. We also examined the use of butylhydroxytoluene ((BHT), 10 µL of 2% BHT solution in the samples), considered by many authors as essential to prevent oxidation reactions leading to artifactual production of MDA [233]. We didn't observe any improvement with BHT. Lastly, we examined if there could be a crosstalk between the MDA-d2 and the standard in a situation where protons might exchange with both deuterium in MDA-d2 and induce an internal contamination. We did not observe any modification in procedural blank when five times more internal standard was injected into the solution (Figure 21).

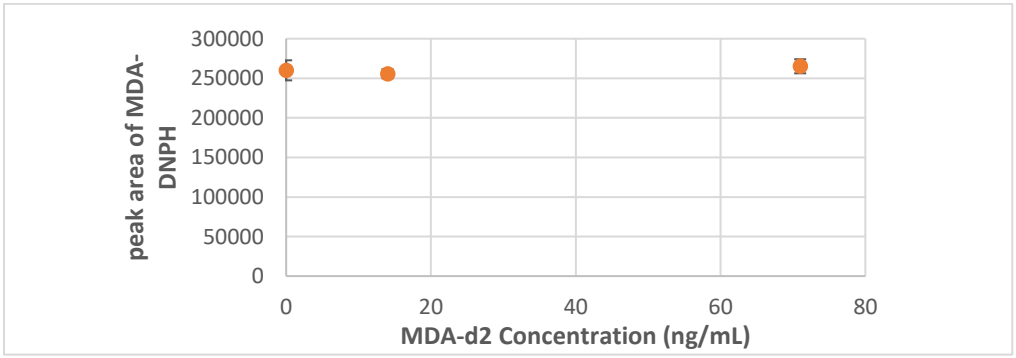


Figure 21. *Effect of MDA-d2 concentration on the peak area of MDA-DNPH. For a standard solution at 0 ng/ml. The error bars correspond to the standard deviation of four measurements*

Nevertheless, we observed that a reduction of the amount of organic solvent in favor of water in the DNPH derivatization solution decreased significantly the signal in the procedural blank (one-way ANOVA p=0.038, data not shown). We also observed that this blank signal increased by 162% by re-using a DNPH solution stored at 4 °C for 1 day (n= 8). These results highlight the need to introduce procedural blanks in the analytical method to control the variation of contamination between batches and to use freshly prepared DNPH solutions. As we could not eliminate this signal, the EBC results had to be corrected with this procedural blank.

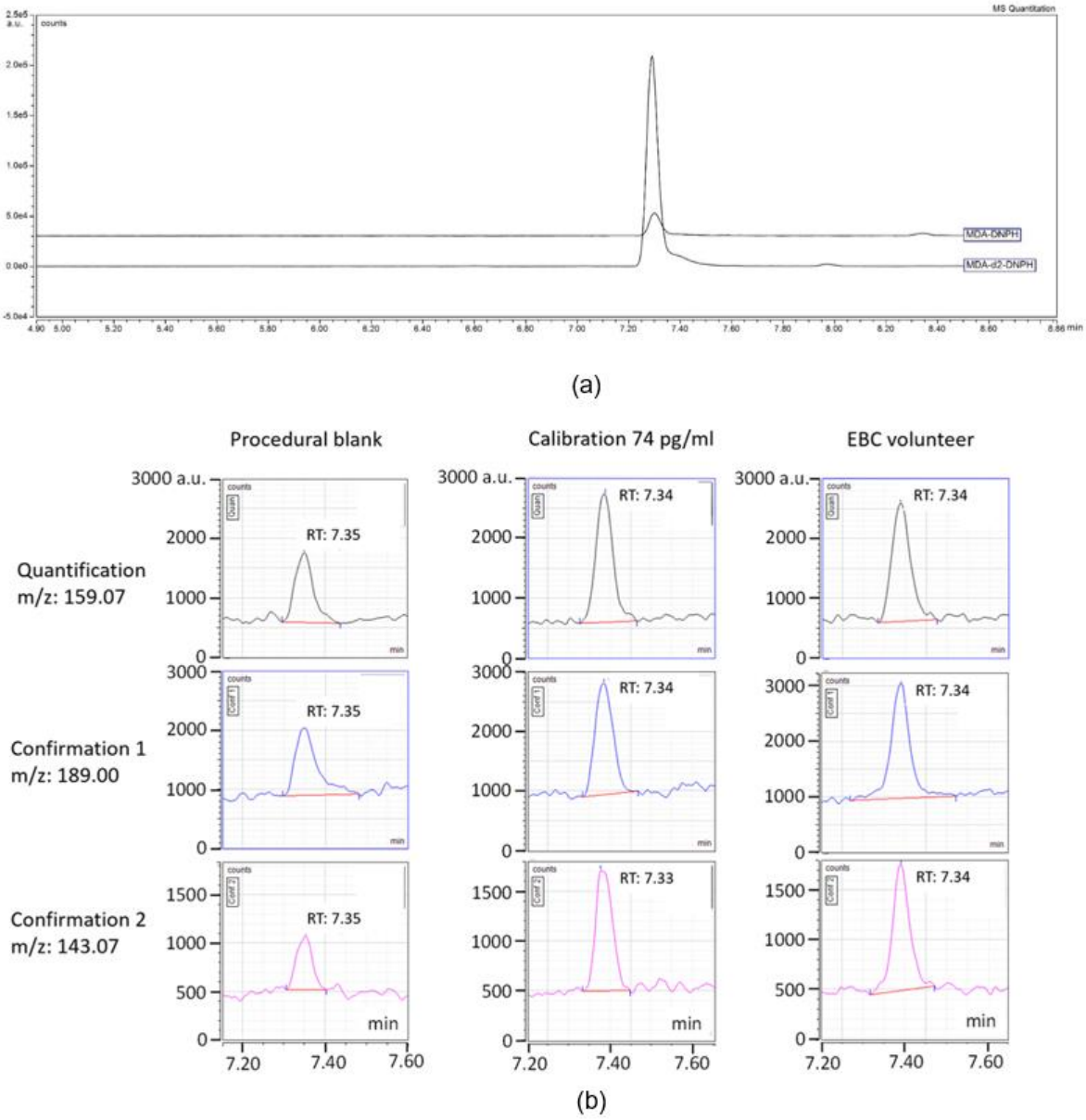


Figure 22. *a) Chromatogram of procedural blank and its internal standard and multi-reaction monitoring (MRM) transitions for MDA b) Partial chromatogram of a procedural blank, of the lowest standard MDA concentration and of a typical EBC sample, with retention times (RT). The units of the y-axis are in arbitrary units, counts per second.*

Method validation and estimation of uncertainty

Table 10 summarizes the performances of the validated method following the FDA/ICH guidelines. The calibration curve, corresponding to the plot of the ratio signal MDA-DNPH / MDA-d2-DNPH as a function of the concentration of the added MDA was linear in the defined concentration range (74-2220 pg/mL) with linear regression coefficients $R^2 > 0.995$ for all series. The slope variability (n=12) was 10.8% whereas a higher variability of 66.1% was determined for the intercept, indicative of a potential strong effect of the blank on the calibration curve. Over the entire standard concentration range, the observed percentage bias of back-calculated MDA concentrations was between 11.1 ± 7.4% (for the lowest concentration) and 3.0 ± 4.5% (for the greatest concentration). The LOD estimated from the error on the intercept was 70 ± 36.5 pg/mL, corresponding to a LOQ of 211 pg/mL. The maximum acceptable deviation observed at this concentration was smaller than 20% of the LOQ, as proposed by the FDA/ICH guidelines (Figure 23). Recovery rates for QC ranged from 92.4 ± 13.0% to 93.5 ± 7.3% for low (211 pg/mL) and high (2258 pg/mL) MDA concentration in EBC respectively. The corresponding repeatability was smaller than 20% for low concentration (211 pg/mL) and 15% for the greatest concentration (2258 pg/mL).

Table 10. *Validation parameters determined for the analysis of MDA in EBC following the FDA/ICH guidelines*

Method characteristics	Expected performance	Observed value	Conclusion
Calibration function (n=12)	-	-	Linear
Concentration range (SD)[1]			
Error for SD 1 = 74 pg. mL^{-1} (n=6)	20%	11.1 ± 7.4%	Verified
Error for SD 2 = 148 pg. mL^{-1} (n=6)	10%	5.9 ± 3.5%	Verified
Error for SD 3 = 370 pg. mL^{-1} (n=6)	10%	2.2 ± 1.1%	Verified
Error for SD 4 = 740 pg. mL^{-1} (n=6)	10%	2.7 ± 2.2%	Verified
Error for SD 5 = 1110 pg. mL^{-1} (n=6)	10%	2.2 ± 1.4%	Verified
Error for SD 6 = 1480 pg. mL^{-1} (n=6)	10%	2.2 ± 1.4%	Verified
Error for SD 7 = 2220 pg. mL^{-1} (n=6)	10%	3.0 ± 4.5%	Verified
LOQ	211 pg. mL^{-1}		
Maximum acceptable deviation	20%	13,1%[2]	Verified
Recovery			
EBC not spiked (n=12)		98.63 pg.mL^{-1}	
EBC level 1 spiked with 12 pg (n=15)	211.2 pg. mL^{-1}	209.1 ± 39.9 pg. mL^{-1}	
EBC level 2 spiked with 24 pg (n=15)	349.3 pg. mL^{-1}	371.4 ± 40.8 pg. mL^{-1}	
EBC level 3 spiked with 62 pg (n=15)	642.2 pg. mL^{-1}	651.2 ± 49.6 pg. mL^{-1}	
EBC level 4 spiked with 124 pg (n=15)	1180.9 pg. mL^{-1}	1161.4 ± 60.4 pg. mL^{-1}	
EBC level 5 spiked with 248 pg (n=15)	2258.3 pg. mL^{-1}	2070.4 ± 175.7 pg. mL^{-1}	
Recovery EBC spiked level 1	90%–110%	92.4 ± 13.0%	Verified
Recovery EBC spiked level 2	90%–110%	106.5 ± 2.8%	Verified
Recovery EBC spiked level 3	90%–110%	101.4 ± 4.0%	Verified
Recovery EBC spiked level 4	90%–110%	98.3 ± 0.9%	Verified
Recovery EBC spiked level 5	90%–110%	93.5 ± 7.3%	Verified
Repeatability			
EBC spiked level 1 (n=15)	20%	9.4%	Verified
EBC spiked level 2 (n=15)	15%	7.9%	Verified
EBC spiked level 3 (n=15)	15%	4.8%	Verified
EBC spiked level 4 (n=15)	15%	4.3%	Verified
EBC spiked level 5 (n=15)	15%	2.7%	Verified
Inter-day precision			
EBC spiked level 1 (n=15)	20%	19.6%	Verified
EBC spiked level 2 (n=15)	15%	8.9%	Verified
EBC spiked level 3 (n=15)	15%	7.0%	Verified
EBC spiked level 4 (n=15)	15%	3.4%	Verified
EBC spiked level 5 (n=15)	15%	9.5%	Verified

[1] between 74 pg. mL^{-1} and 2220 pg. mL^{-1}
[2] With a confidence interval of 95%

The relative error of back-calculated EBC concentrations, related to their targeted concentrations (accuracy) is shown in the form of a box-and-whisker plot in Figure 23. The error for each concentration was comprised between the acceptance limits (fixed at 20%), demonstrating the validity of the method for the considered concentration range. The acceptance limits of 20% was exceeded for only two results that can be considered as potential

outliers at the concentration of 211.2 pg/mL and the concentration 349.3 pg/mL respectively, confirming the LOQ of 211 pg/mL.

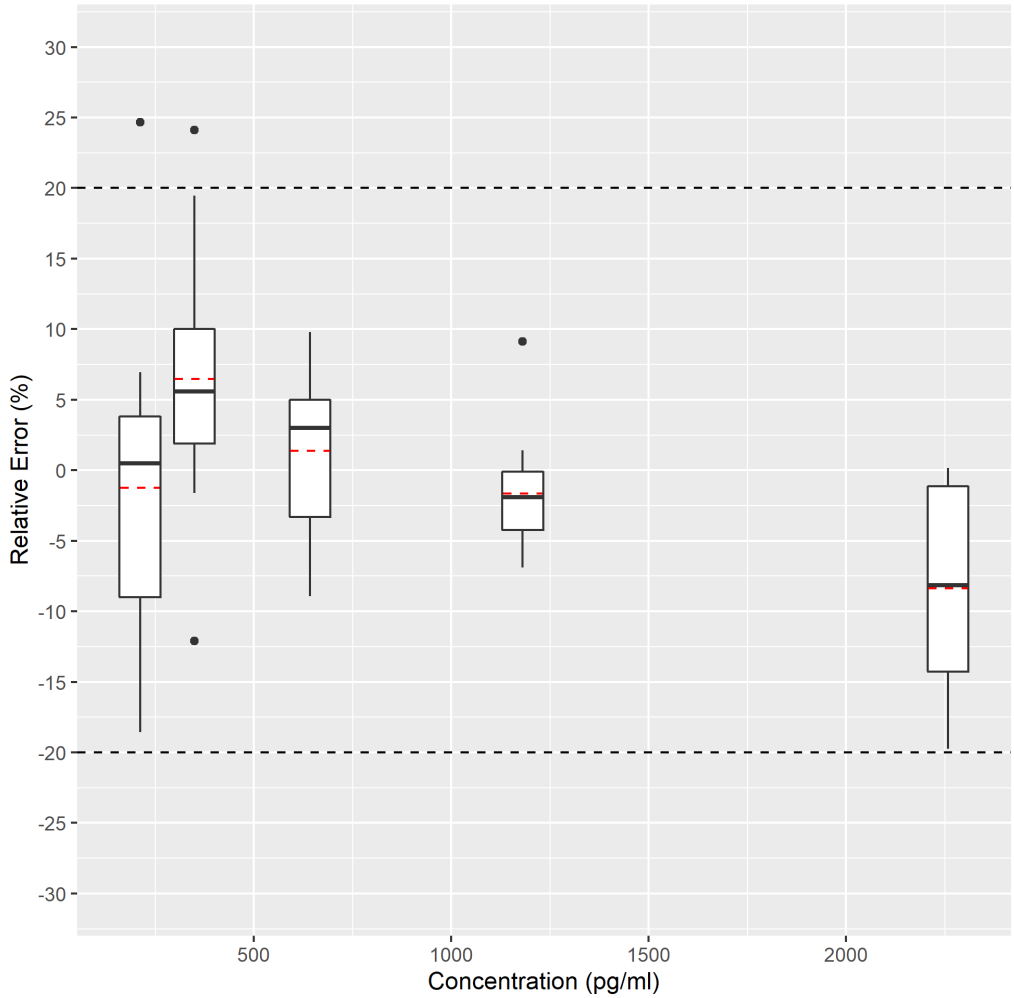


Figure 23. *Accuracy profile for MDA analysis in EBC represented in the form of box-and-whisker plot. The figure denotes the measured relative error compared to the targeted spiked concentration. The black central line represents the median value (50th percentile) whereas the red dashed lines represent the average value. The upper and lower whiskers correspond to the adjacent values as defined by Tukey [234]. Data points falling outside the adjacent values can be considered as potential outliers and are plotted separately. The external dashed lines correspond to the acceptance limits (fixed at 20%).*

Matrix effect was examined by comparing the slope of the calibration curves in water and in spiked EBC, using an unpaired t-test. No statistically significant difference was observed between the slopes from calibration standard ($2.4.10^{-4}$ ± $0.31\ 10^{-4}$) and the EBC samples ($2.3.10^{-4}$ ± $0.31\ 10^{-4}$) (unpaired t-test $p>0.05$, $n=12$).

Standard solutions of MDA at 5.4 µg/mL and IS at 567 µg/mL as well as QC EBC samples were observed to be stable for at least 8 months at −80 °C, with an observed decreased concentration < 4% after that storage duration. Once derivatized, the MDA-DNPH compound was stable for at least 48 h when stored in an auto-sampler at room temperature (23 °C).

Uncertainty

The contribution of the three identified components to the uncertainty was determined using the data generated during the method validation process for EBC QC samples (Figure 24). The precision (repeatability) was the largest contributor to the uncertainty, reaching an average value of about 5.2%.

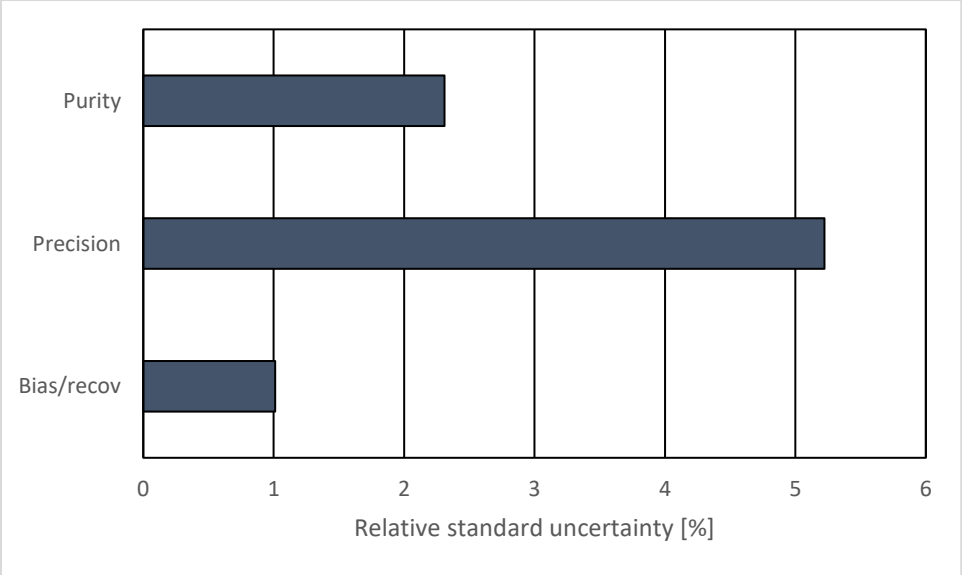


Figure 24. *The relative standard uncertainty contribution for each parameter considered in the uncertainty measurement*

Figure 25 presents the calculated expanded uncertainty for the different MDA concentration in EBC, using a coverage factor of 2. As expected, the lowest uncertainty (≤ 10%) was observed for MDA concentrations above 650 pg/mL, whereas it increased to 23% for MDA concentrations near the LOQ.

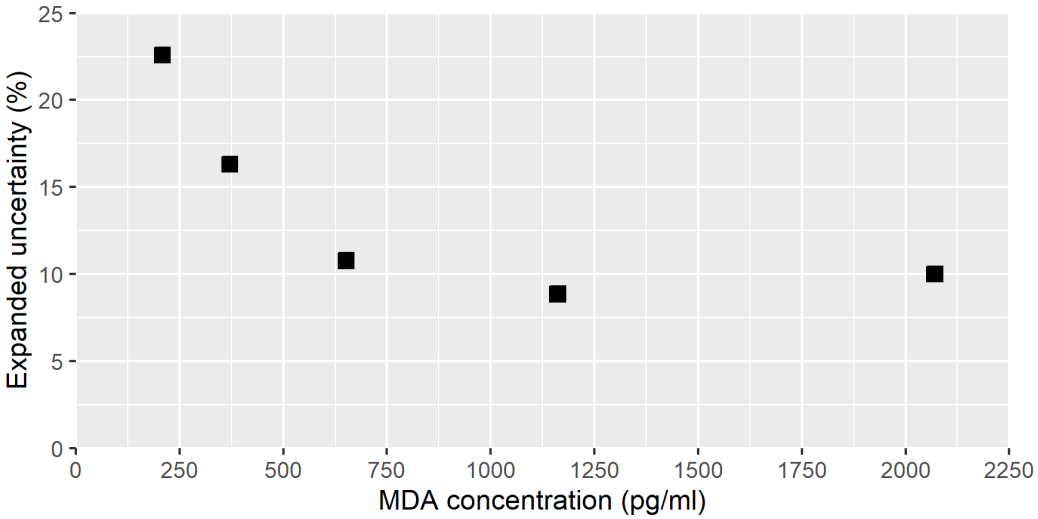


Figure 25. *The calculated expanded uncertainty, using a coverage factor of 2, as a function of the MDA concentration in EBC*

Levels of MDA in EBC of healthy adult workers

Table 11 presents the concentrations of MDA in EBC of nine workers, splitted in three different occupations. For all the samples, 19% of MDA concentrations were below the LOD (70 pg/mL), 63% were included between the LOD and LOQ (211 pg/mL) and 18% were above the LOQ (Figure 26). Concentrations of MDA were relatively low, with a highest concentration measured at 886 pg/mL.

Table 11. *Concentrations of MDA in 164 EBC samples from nine healthy individuals from three different professional categories, collected over two months*

	Professional category 1			**Professional category 2**			**Professional category 3**		
Participant N°	1	2	3	4	5	6	7	8	9
MDA mean concentration [pg/mL] (number of replicates)	130[a] (19)	255 (19)	393 (20)	75[a] (18)	110[a] (18)	170[a] (17)	<LOD (18)	<LOD (18)	155[a] (17)
Minimum [pg/mL]	78[a]	<LOD	95[a]	<LOD	<LOD	115[a]	<LOD	<LOD	85[a]
Maximum [pg/mL]	339	597	886	154[a]	236	345	141[a]	229	419
Intra-individual variability [%] (number of replicates)	46 (19)	60 (19)	68 (20)	39 (18)	35 (18)	33 (17)	47 (18)	85 (18)	49 (17)
Inter-individual variability [%][b]		51			41			57	

[a]: MDA concentrations were between the LOD (70 pg/mL) and LOQ (211 pg/mL)

[b]: for the same occupation (n=3)

The intra-individual MDA variability was between 33-85%, whereas the inter-individual variability was slightly smaller (41-57%). These variabilities must be compared to the estimated analytical uncertainty of about 23% for low (≈200 pg/mL) MDA levels in EBC (Figure 25).

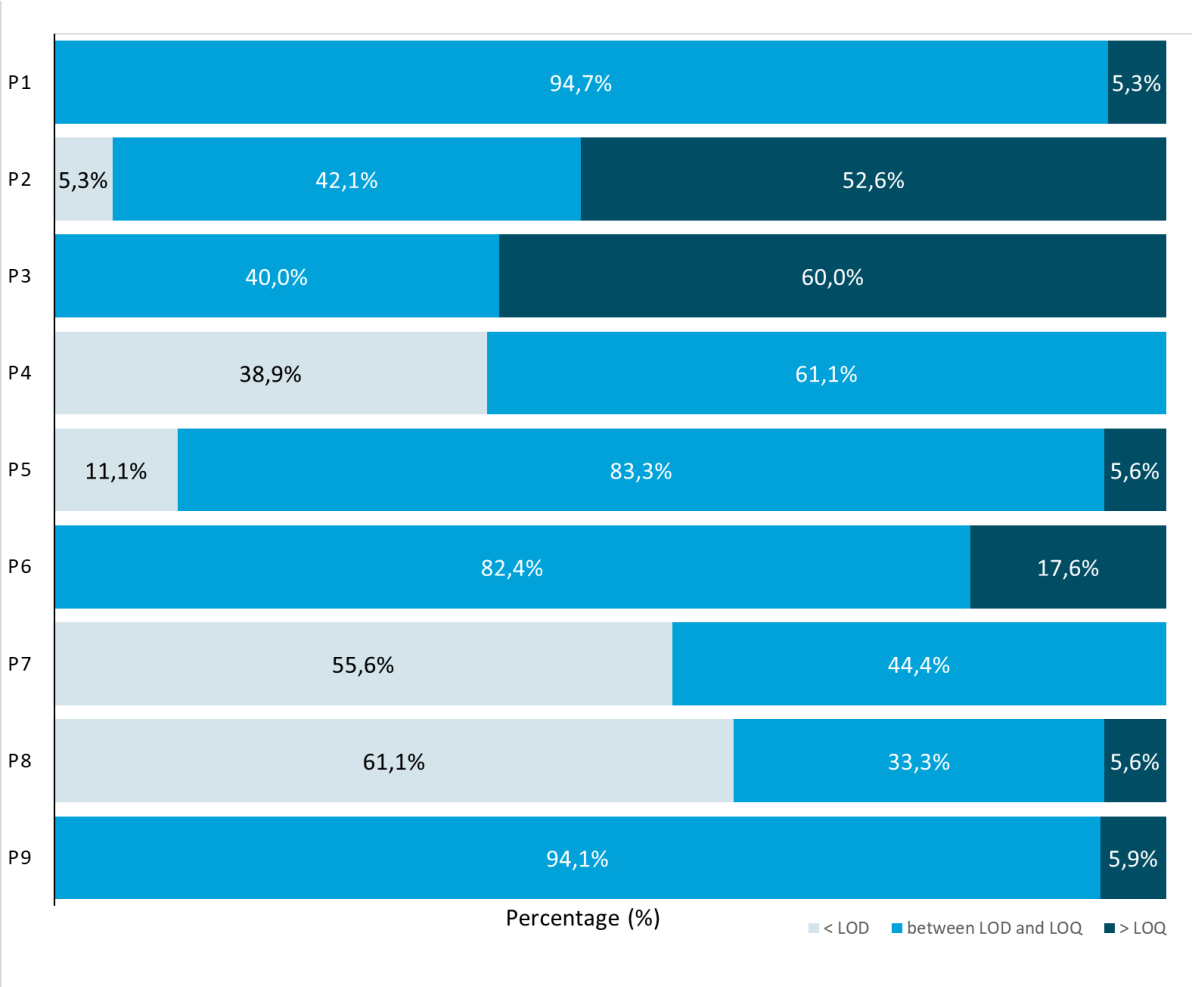


Figure 26. *Stacked bar graph representing the percentage of values per participant below the LOD (light blue), between the LOD and LOQ (blue) and above the LOQ (dark blue)*

To assess the advantages of our improved analytical method for epidemiological studies, we assumed: (1) a study design where each subject is sampled once; (2) log-normal distributions for MDA concentrations in EBC (as observed in our dataset); (3) a CV of 30% (worst case scenario) corresponding to an intra-individual variance on the log scale of 38% (intra-subject variability of 50%), and a total variance of 19% (inter-subject variability of 50%). We found the analytical variability to be far lower than the inter-subject variability, and conclude that the proposed analytical method is able to detect relevant inter-subject differences in epidemiological studies.

2. Development of method for 8-OHdG/8-isoprostane

a. Context

This method aimed at development and optimization of a method to quantitate simultaneous 8-OHdG and 8-isoprostane in exhaled breath condensate, where the analytes are present at an exceptionally low concentration. In this study, the potential usefulness of LC-MS/MS procedure for the accurate qualitative analysis of EBC is investigated. Because of the limited volume and the much-diluted nature of the EBC sample, Vacuum concentration is used as a pre-concentration technique.
These types of results have never been reported in the scientific literature and illustrates well the future challenges for the characterization of trace biomarkers in very dilute matrices like EBC in order to extract meaningful results linking to human health outcomes.

This work was described in the scientific manuscript entitled "Quantification of 8-OHdG and 8-isoprostane in exhaled breath condensate " **published** as original article in the Special Issue "The 10th Anniversary of Antioxidants: Past, Present and Future" of the journal Antioxidants.

b. Materials and Methods

Reagents-chemicals

8-OHdG (≥ 98%) (2-amino-9-[(2R,4S,5R)-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,7-dihydropurine-6,8-dione) in a solid form was obtained from Merck (Buchs, St. Gallen, Switzerland). [15N5]-8-OHdG as internal standard (IS) was obtained from Cambridge Isotope Laborato-ries (Tewksbury, MA, USA). 8-Isoprostane ((5Z,8β,9α,11α,13E,15S)-9,11,15-trihydroxyprosta-5,13-dien-1-oic acid) in a solution ≥ 95%, and its deuterated IS 8-Isoprostane-d4 ((5Z,8β,9α,11α,13E,15S)-9,11,15-trihydroxyprosta-5,13-dien-1-oic-3,3,4,4-d4 acid), were obtained from Cayman Chemical (Ann Arbor, MI, USA). HPLC grade methanol (≥ 99.9%) was obtained from Merck (Buchs, Switzerland). LC-MS grade solvents; methanol (≥ 99.95%) and acetonitrile (≥ 99.9%) were obtained from Carlo Erba Reagents (Chaussée du Vexin, Val de Reuil, France). Butylated hydroxytoluene (BHT) ≥ 99% was obtained from Sigma-Aldrich Produktions GmbH (Steinheim Germany). LC–MS grade acetic acid was obtained from Honeywell (Seelze, Germany). Ultrapure water was produced in our laboratory with a Milli-Q Advantage water purification system (18.2 MΩ.cm at 25 °C, < 3 ppb total organic carbon).

Preparation of standards

Stock solutions at 1 mg/mL of 8-OHdG and 1 mg/mL 8-isoprostane in H_2O/MeOH (8:2) were prepared and stored at −20 °C. A mother stock solution for each analyte (5000 ng/mL) was prepared by diluting 50 µL of the stock solution of 8-OHdG or 8-isoprostane 1 mg/mL with ultrapure water in a volumetric flask of 10 mL. This stock solution was stored at 4 °C. [^{15}N5]-8-OHdG at 500 ng/mL was prepared by diluting 10 µL of [^{15}N5]-8-OHdG 25 µg/mL in a conical glass vial with screw cap containing 490 µL of ultrapure water. 8-isoprostane d4 at 500 ng/mL was prepared by diluting 10 µL of 8-isoprostane d_4 100 µg/mL in a glass tube containing 1990 µL of ultrapure water. Final solutions were vortexed and stored at 4 °C. These stock standard solutions can be used at least 3 months without noticeable concentration variation. These different stock solutions were mixed and diluted in ultrapure water to prepare two daily working solutions of 8-OHdG and 8-isoprostane (500 pg/mL each) and an internal standard mix (Mix IS) of [^{15}N5]-8-OHdG and 8-isoprostane d_4 (2.5 ng/mL each). Five calibration standards (final concentrations of 0, 15, 50, 100, and 300 pg/mL) were obtained by diluting the working solutions as described in Table 12.

Table 12. *Description of the standard preparation with the corresponding concentrations*

Label	*volume of working solution at 500 pg/mL [µL]*	*Volume of H_2O [µL]*	*Volume of working solution of IS 2,5ng/ml [µL]*	*Volume of BHT [µL]*	*Total volume [µL]*	*Final concentration Std [pg/ml]*	*Final concentration IS[ng/mL]*	*BHT (mg/mL)*	*Container*
std 0	0	800	200	10	1010	0	0,495	0,099	polypropylene vial
Std 15	30	770	200	10	1010	15	0,495	0,099	polypropylene vial
Std 50	100	700	200	10	1010	50	0,495	0,099	polypropylene vial
Std100	200	600	200	10	1010	99	0,495	0,099	polypropylene vial
Std300	600	200	200	10	1010	297	0.495	0.099	polypropylene vial
Before concentration									
H20 0	0	985	15	10	1010	0	0,037	0,099	Eppendorf
H20 5	10	975	15	10	1010	5	0,037	0,099	Eppendorf
H20 15	30	955	15	10	1010	15	0,037	0,099	Eppendorf
H20 25	50	935	15	10	1010	25	0,037	0,099	Eppendorf
H20 50	100	885	15	10	1010	50	0,037	0,099	Eppendorf
						After concentration and reconstitution in 75µL			
						0	**0,495**	**1,320**	polypropylene vial
						67	**0,495**	**1,320**	polypropylene vial
						200	**0,495**	**1,320**	polypropylene vial
						333	**0,495**	**1,320**	polypropylene vial
						666	**0.495**	**1.320**	polypropylene vial

Std = not evaporated standards; H20 = standards that will be evaporated.

EBC samples preparation

EBC samples used for validation and quality control (QC) were collected from two healthy non-smoking voluntary adult from our laboratory. EBC was collected according to recommendations by the American Thoracic Society/European Respiratory Society Task Force [180]. The EBC collection device (TurboDeccs, Medivac, Parma, Italy) operated at -10 °C and was equipped with a salivary trap [235] and a disposable polypropylene plastic collection system (DECCS 14 ST kit). Each volunteer sat comfortably, wore a nose-clip, and breathed in through the mouthpiece for 20 min. The collected EBC liquid was transferred into a cryovial (Sarstedt, Nümbrecht, Germany) and stored at −20 °C (for one day for logistical reasons) then transferred to −80 °C until analysis (within 8 months).

Analytical procedure

i. Sample preparation

A concentration step is necessary due to the low concentrations of these oxidative stress biomarkers in EBC (*see Part 5*). One ml of the standard or EBC sample was introduced in a plastic tube (1.5 mL), followed by 10 µL of BHT 10 mg/mL and 15 µL of Mix IS 2.5 ng/mL. BHT is considered as essential to prevent further in vitro oxidative formation of 8-isoprostane [236]. without impacting the 8-OHdG concentration in EBC [237]. The mixture was then vortexed and put in a concentrator (Speedvac SVC-100H, Savant In-struments Inc, Farmingdale, NY, USA) under vacuum with heat (set at 45 °C) and pressure (range 10–22 mbar). The complete evaporation of the water took 6.5 h. Another concentration step using lyophilization (freeze-

drying) (Freezone 1, Labconco, Fort Scott, KS, USA) at 0.06 mbar and < −50 °C at the condenser for 8 h was also tested. With both techniques, the residue was dissolved in 75 µL of water acidified with 0.1% acetic acid (LC-MS grade) (corresponding to a concentration factor of 13.3), vortexed and sonicated for 5 min. The final solution was transferred into a polypropylene vial (300 µL) (Macherey-Nagel AG, Oensingen, Switzerland) before chemical analysis. Standard solutions (concentration of 0, 5, 15, 25, and 50 pg/mL; Table 12) were included with each series of samples and followed the same procedure. These standards were used for quantification, whereas the non-concentrated solutions (std 0–300 pg/mL) were used as a reference. Each sample (50 µL) was injected into the LC–MS system. EBC spiked with both analytes at 5 and 50 pg/mL were prepared and served as quality controls (QC).

ii. Assessment of type of sample concentration

Two concentrating methods based either on lyophilization or using vacuum concentration were performed. Experiments consisted of concentrate with five standards solution in water (0–50 pg/mL of each analyte) or five spiked EBC samples (0–50 pg/mL of each analyte) with each technique. Vials of either glass or low-binding plastic used during this concentration step were tested. By plotting the signal obtained for each solution as a function of the concentration, a linear regression characterized by its slope was drawn through these points. We compared these different slopes with the corresponding standard solutions that have not undergone the concentration step.

iii. EBC material surface absorption and interferences

Adsorption phenomena on material surfaces have been reported for biomarkers in EBC, particularly for eicosanoids [238-240]. We thus used a procedure described by Tufsesson et al., [241] to coat all plastic surfaces with 0.01% Tween 20 (Sigma Aldrich, Burlington, VT, USA) for 30 min. This included all the pieces of the EBC kit (TurboDeccs sample container and tubing). After this treatment, the material was carefully rinsed three times with ultrapure water and then left to dry in an oven at ambient temperature. An EBC collection was then performed on this material by following the same procedure as previously described. An EBC collection was also simulated by placing 2 mL of a 5 pg/mL standard in the collection tube and leaving it in contact with the plastic for 30 min with stirring. The recovery rate compared to what was originally injected was thus assessed. The effect of using glass or plastic containers (low-binding plastic tubes) during the concentration step using lyophilization or Speedvac was also examined.

We tested a centrifugal concentrator system using a membrane of cellulose triacetate with a cut-off of 5000 Dalton (Vivaspin TCA 5kD, Sartorius, VWR, Dietikon, Switzerland) to avoid possible interferences from proteins during the nebulization in the LC–MS system [242,243].

EBC samples was spiked with the two biomarker standards at 15 pg/mL, and centrifuged (Vivaspin at 4000 rpm for 90 min). The filtrate was concentrated to dryness using the vacuum concentrator and the residue dissolved in 0.1% acetic acid as described previously.

iv. Analytical conditions

The target biomarkers were analyzed with an ultra-high pressure liquid chromatography (LC) system (Dionex Ultimate 3000) equipped with a C18 column (Zorbax Eclipse Plus 2.1 × 100 mm, 1.8 µm, Agilent, Morges, Switzerland) and operated at 50 °C. The injection volume was 50 µL. The solvent gradient (flow rate of 0.25 mL/min) was a combination of eluent A (H2O with 0.1% acetic acid) and eluent B (MeOH/ACN 7:3 with 0.1% acetic acid). The LC was operated with the following program: 100% A for 2 min, decreasing to reach 10% over 8 min, held for 4.5 min, then increased to 100% A in 1 min, and held for 6.5 min. A Triple-Stage Quadrupole MS (TSQ Quantiva Thermo Scientific, Reinach, Switzerland) with electrospray ionization (ESI) was used for detection (instrument parameters shown in Table 13). All data acquisition and processing were accomplished using the Thermo Scientific Chromeleon software.

Table 13. *MS transitions and instrumental conditions for 8-OHdG and 8-isoprostane analysis. The vaporizer temperature was 350°C with the ion transfer tube set at 390°C. The argon gas pressure was set at 1.5 mTorr*

Compounds	Polarity	Mass transitions [m/z]	Spray voltage [V]	Collision energy [V]	RF lens [V]
8-OHdG	positive	284 → 140	3700	28.8	37
		284 → 168		10	37
		284 → 243		10.2	37
[^{15}N5]-8-OHdG	positive	**289 → 173**	3700	10	40
8-isoprostane	negative	**353 → 193**	3400	25	80
		353 → 291		20	80
		353 → 309		20	80
8-isoprostane-d_4	negative	**357 → 197**	3400	25	78

Mass transitions in bold are quantification transitions, others are confirmation transitions.

Method validation and applicability

i. Method validation

The optimized method was validated by considering linearity, limit of detection (LOD), limit of quantification (LOQ), intra-day and inter-day precisions, recovery and matrix effects according

to FDA/ICH guidelines [221]. The LOD and LOQ were determined by dividing the error on the origin for the calibration standard by the calibration slope. Different pooled EBC samples were used to determine recovery and repeatability. The calibration curve was determined by plotting the peak area ratio standard/IS as a function of the concentration of the added standard. The final concentration of each oxidative stress marker in EBC was calculated based on the calibration curve obtained with the standard treated identically as for the sample.

$$[\text{Analyte}]_{\text{EBC}} = \frac{\text{Ssple}-\text{B}}{\text{A}} \times \frac{1}{13.33}$$

Where:

[Analyte]$_{EBC}$: Concentration of the considered marker (8-OHdG or 8-isoprostane) in EBC [pmol/ml].

Ssple: Peak area of the sample/ peak area of the IS.

A: Slope of the calibration curve (Peak area standard/peak area IS) = f[concentration standard].

B: Ordinate at the origin of the calibration curve.

1/13.33: Concentration factor, corresponding to the ratio between the initial 1 ml EBC and the final 75 µL of the final sample after vacuum concentrator treatment.

ii. Study population

The suitability of the validated method was assessed using EBC samples from 26 workers from the same workplace. These samples were selected from a study sample of 303 RATP workers. The RATP cohort was stratified based on their spirometry results, distinguishing healthy (n=10), asthmatics (n=7), and COPD presenting subjects (n=9) [244]. Informed consent was obtained from all subjects and the study was approved by the French Personal Protection Committees South-Est II (N°2019-A01652 55) and South-Est IV (N°2020-A03103-36). The demographic characteristics of participants are presented in Table 14.

Investigators were kept blind with respect to the health status, which was only revealed at the statistical analysis stage. The hypothesis I tested was that workers with asthma or COPD would present higher concentrations of oxidative stress biomarkers in EBC compared to the healthy workers.

Table 14. *Summary of participant demographics (n=26)*

	Healthy	**Asthmatics**	**COPD**
Number of subjects (male/female)	10 (6/4)	7 (3/4)	9 (6/3)
Age Mean years ±SD (range)	51 ± 5,2 (44-60)	47 ± 5,2 (40-57)	54 ± 5,8 (41-60)
BMI Mean kg/m2 ±SD (range)	25 ± 3,7 (20-32)	25 ± 2,4 (22-28)	24 ± 4,9 (19-34)
FEV_1/FVC ratio	0,771 ± 0,04 (0,713-0,825)	0,665 ± 0,04 (0,597-0,697)	0,611 ± 0,06 (0,501-0,675)
Smokers (%)	30%	28%	66%

SD = standard deviation; BMI = body mass index; COPD=Chronic obstructive pulmonary disease; FEV 1 = forced expiratory volume in one second; FVC = forced vital capacity.

Statistical analysis

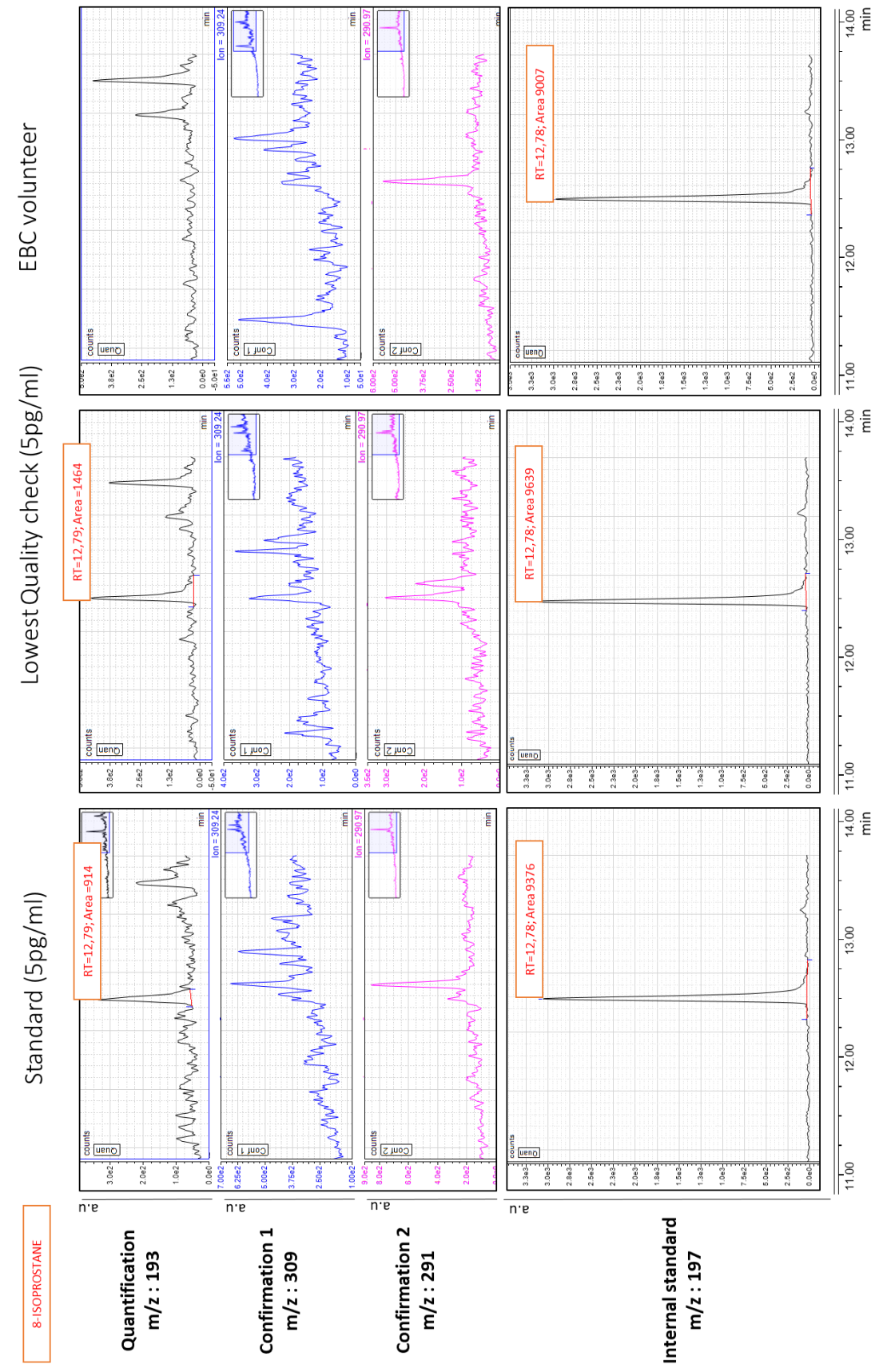
Descriptive analysis were performed with the built-in statistical functions in Microsoft Excel version 2016, whereas t-test calculations were performed with the R software (R version 4.0.2,). Results are expressed as means± SD.

c. Results

8-OHdG and 8-isoprostane analytical performance

Figure 27a and b give typical chromatograms of 8-isoprostane and 8-OHdG, respectively. These chromatograms were obtained after injection of 50 µL of a low standard level at 5 pg/mL, the lowest EBC quality check sample (5 pg/mL) and concentrated EBC sample collected from a healthy volunteer using the optimized conditions.

(a)


8-ISOPROSTANE
Standard (5pg/ml)
Lowest Quality check (5pg/ml)
EBC volunteer
RT=12,79; Area =914
RT=12,79; Area =1464
RT=12,78; Area 9376
RT=12,78; Area 9639
RT=12,78; Area 9007
Quantification m/z : 193
Confirmation 1 m/z : 309
Confirmation 2 m/z : 291
Internal standard m/z : 197
a.u
min
11.00
12.00
13.00
14.00

(b)

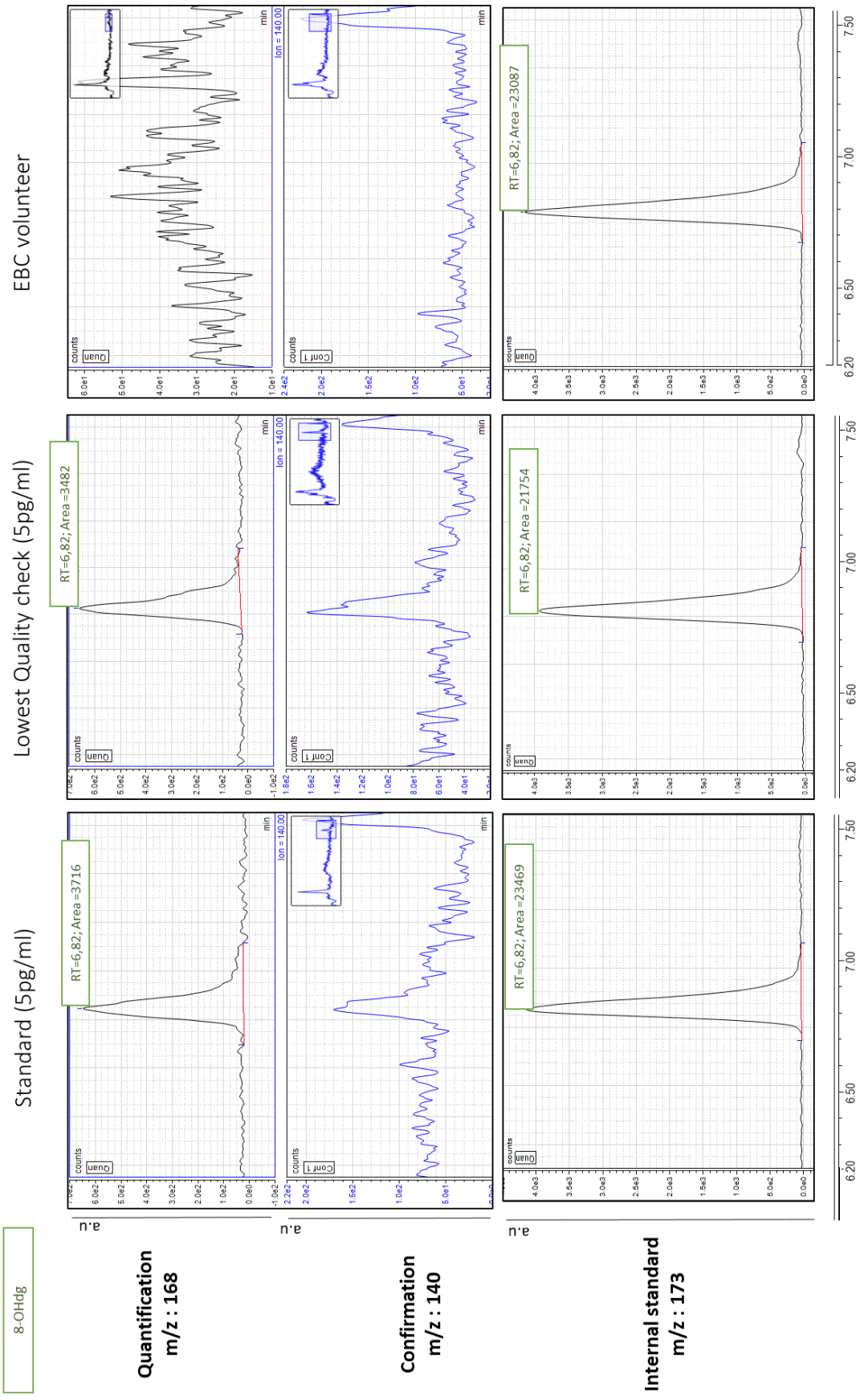

8-OHdg
Standard (5pg/ml)
Lowest Quality check (5pg/ml)
EBC volunteer
Quantification m/z : 168
Confirmation m/z : 140
Internal standard m/z : 173
RT=6,82; Area =3716
RT=6,82; Area =3482
RT=6,82; Area =23469
RT=6,82; Area =21754
RT=6,82; Area =23087
a.u
counts
min
Quan
Conf 1
Ion = 140.00

Figure 27. (a, b) *Partial chromatogram of a standard injection at 5pg/mL (left), of the lowest quality check (spiked EBC at 5 pg/mL, middle) and of a typical EBC sample (right), with retention times (RT) for 8-isoprostane and 8-OHdG, respectively. The units of y-axis are in arbitrary units, counts per second. (a, b) are chromatograms obtained from the same healthy volunteer.*

Influence of sample preparation process

We tested two concentrating methods based either on lyophilization or using vacuum concentration. Figure 28 represents the mean slope of the linear regression based on five concentrated solutions for a minimum of two repetitions. We observed that glass containers needed a longer duration (about 9 h) to reach complete dryness compared to low binding plastic tubes (about 6.5 h).

We observed a significant and systematic decreased slope for 8-OHdG in glass vial (20-35%) and a statistically significant decreased slope (H_20 lyoph glass p= 0.022; EBC lyoph glass p=0.033; EBC lyoph plastic p= 0.041) for 8-isoprostane (35-40%) when the lyophilization was conducted at low pressure (0.06 mbar) compared to the standard without the concentration step. On the contrary, when using the vacuum concentration at a higher pressure (16 mbar), the slope was similar (8-isoprostane) or even significantly higher (8-OHdG) (p=0.007) to that obtained without the concentration step. Nevertheless, this increase disappear when the value is related to the SI concentration. Based on these results, we selected the vacuum concentration method for our sample preparations.

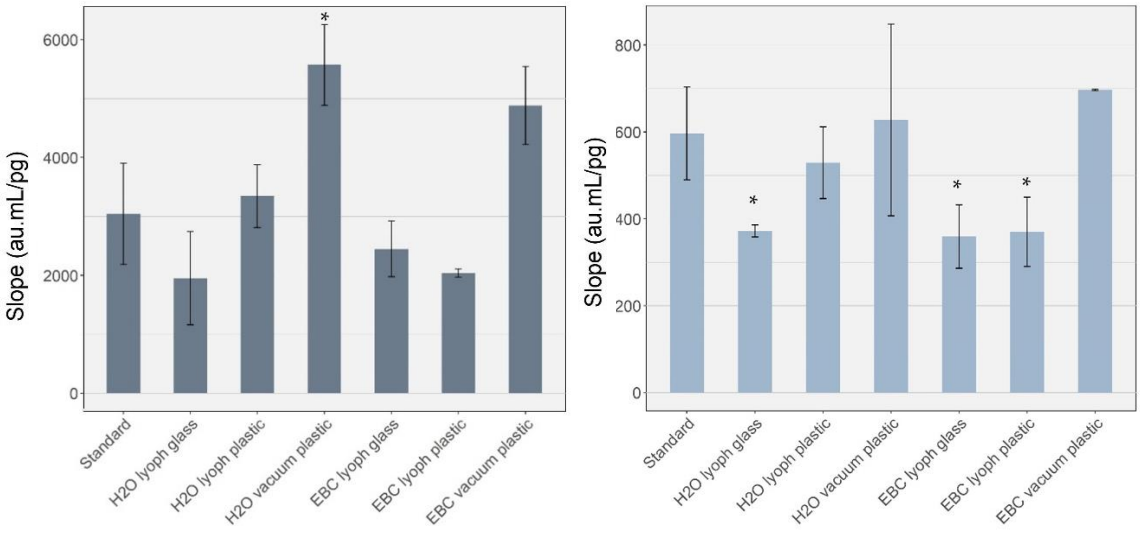


Figure 28. *Slope signal (not corrected with IS) of the calibrations (minimum of 5 levels) obtained with water and EBC after lyophilization (lyoph) or vacuum concentration, in comparison with standard solutions without any concentration step for the biomarker a) 8-OHdG and b) 8-isoprostane, respectively. Error bars correspond to a minimum of two independent repetitions. Au= Arbitrary unit; Lyoph = lyophilization; standard (= non-concentrated standard); H2O lyoph (=concentrated standard).* Statistically significant difference (p< 0.05) compared to the standard without the concentration.*

Effect of protein purification

Proteins are present in EBC in relatively high concentrations (typical range 0.76-107.7 µg/mL EBC [245]). We tested a clean-up procedure using a centrifugal concentrator system to remove high molecular weight proteins. This treatment had a strong impact on 8-OHdG with a two-fold decreasing signal (p= 0.001; n=11) (Figure 29). Such a decrease can be attributed to the strong adsorption of 8-OHdG on the cellulose triacetate membrane [246]. 8-isoprostane on the contrary, presented an increased signal of about 20% compared to the EBC sample without the concentration step. By washing the membrane with water before the sample treatment, we observed a decreased signal, similar to the one without centrifugation. This result suggests that an interfering compound soluble in water was present on the membrane, increasing artificially the signal of 8-isoprostane when the centrifugal concentrator system was used. Based on these results, we decided not to use such a clean-up procedure.

Matrix effects were evaluated by comparing the slope of the calibration standards with the one obtained with the spiked EBCs at the same concentrations and without IS corrections using an unpaired t-test. No statistically significant difference was observed between the slopes from calibration standard not corrected by IS (5569 ± 684, 627 ± 220 for 8-OHdG and 8-isoprostane,

respectively) and the EBC samples (4880 ± 661, 696 ± 1.53) for 8-OHdg and 8-isoprostane, respectively) (unpaired t-test, 8-OHdG p=0.197; 8-isoprostane p=0.831, n=4).

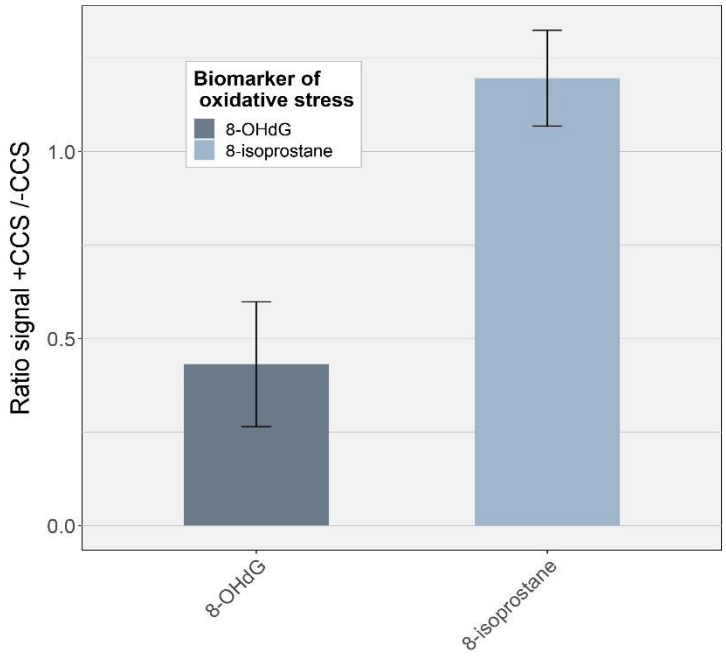


Figure 29. *Effect of using the centrifugal concentrator system on the signal of both analytes in EBC. Bars correspond to standard deviation of three independent repetitions. CCS= centrifugal concentrator system*

Effect of the coating

Experiments of Tufvesson [241] showed that coating disposable polypropylene device with the detergent Tween 20 increased significantly the 8-isoprostane recovery. Nevertheless, we did not observe a difference on 8-OHdG and 8-isoprostane concentrations on our experiments comparing coated and not-coated systems (Figure 30).

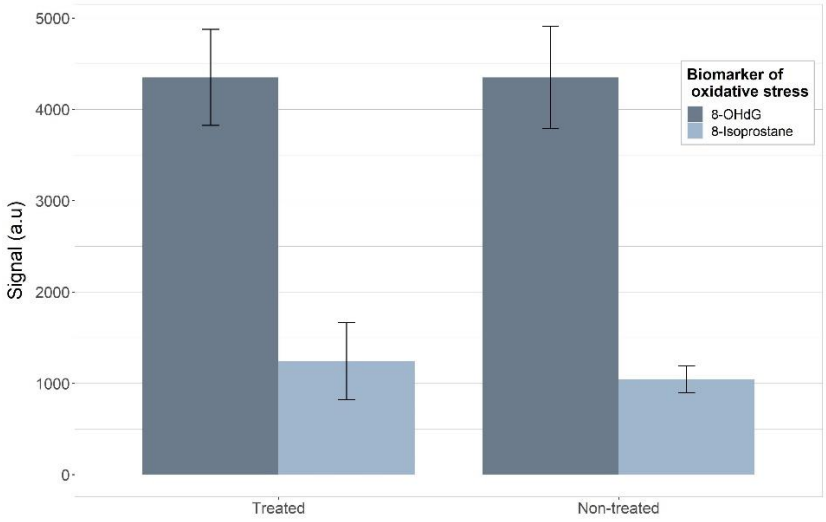


Figure 30. *Effect of Tween-20 treatment on material surface on the signal (not corrected with IS) of 8-OHdg and 8- isoprostane. Error bars correspond to a minimum of nine independent repetitions.*

Method validity

The characteristics of the method are given in Table 15 for 8-OHdG and Table 16 for 8-isoprostane. The LOD was 1 pg/mL EBC for 8-isoprostane and 0.5 pg/mL EBC for 8-OHdG. The recovery was between 90-110%. The repeatability for the two biomarkers was smaller than 20% for at the lowest concentration (5 pg/mL) and smaller than 6% for the higher concentration (15 pg/mL). The criteria for linearity were assessed by means of the coefficient of determination (R^2), which was always above 0.99 in all analyses.

Table 15. *Main figures of merit of the analytical method for 8-OHdG in EBC*

Method characteristics - 8OHdG	**Expected performances**	**Observed values**	**Conclusion**
Calibration			
Function			linear
Concentration range [pg/ml]	0-1333		
Bias for std. 67 pg/ml	>15%	6.9%	Verified
Bias for std. 200 pg/ml	10%	4.9%	Verified
Bias for std. 333 pg/ml	10%	1.3%	Verified
Bias for std. 666 pg/ml	10%	1.0%	Verified
Bias for std. 1333 pg/ml	10%	0.4%	Verified
LOQ (a-priori estimate)	22 pg/ml (ech conc speedvac)		
Maximum accepted range	±13.2 pg/ml =60%*22 pg/ml		
Upper accepted value [pg/ml]	35.2	27.1	Verified
Lower accepted value [pg/ml]	8.8	23.5	Verified
LOD (1/3 LOQ) [pg/ml]		7.30	Verified
LOD in EBC [pg/ml]		0.5	Verified
Recovery			
Value EBC non spiked [pg/ml]	0.00		
EBC spiked 1 [pg/ml]	5.00	4.8	
EBC spiked 2 [pg/ml]	15.00	14.0	
Recovery spike 1 [%]	[90 - 110]	98±10	Verified
Recovery spike 2 [%]	[90 - 110]	89±8	Verified
Repetability (CV)			
EBC non spiked	< LOD	n.d.	
EBC spiked 1 (5 pg/ml)	20%	19.8	Verified
EBC spiked 2 (15 pg/ml)	10%	1.0	Verified
Accuracy			
Reference value EBC spiked 1 [pg/ml]	5.00		
u_{ref}	2.0		
Maximum accepted difference	25%		
Normalized range	2	0.14	Verified
Maximum accepted range [pg/ml]	6.25	6.02	Verified
Minimum accepted range [pg/ml]	3.75	4.53	Verified
Reference value EBC spiked 2 [pg/ml]	15.00		
u_{ref}	2.0		
Maximum accepted difference	10%		
Normalized range	2	-0.52	Verified
Maximum accepted range [pg/ml]	16.50	14.82	Verified
Minimum accepted range [pg/ml]	13.50	13.10	Not verified

Table 16. *Main figures of merit of the analytical method for 8-isoprostane in EBC*

Method characteristics - 8-isoprostane	**Expected performances**	**Observed values**	**Conclusion**
Calibration			
Function			linear
Concentration range [pg/ml]	0-1333		
Bias for std. 67 pg/ml	>15%	10.0%	Verified
Bias for std. 200 pg/ml	10%	-5.9%	Verified
Bias for std. 333 pg/ml	10%	4.5%	Verified
Bias for std. 666 pg/ml	10%	-2.6%	Verified
Bias for std. 1333 pg/ml	10%	1.0%	Verified
LOQ (a-priori estimate)	40 pg/ml (ech conc speedvac)		
Maximum accepted range	±24 pg/ml =60%*40 pg/ml		
Upper accepted value [pg/ml]	63.5	27.9	Verified
Lower accepted value [pg/ml]	15.9	18.3	Verified
LOD (1/3 LOQ) [pg/ml]		13.2	Verified
LOD in EBC [pg/ml]		1.0	Verified
Recovery			
Value EBC non spiked [pg/ml]	0.00		
EBC spiked 1 [pg/ml]	5.00	5.4	
EBC spiked 2 [pg/ml]	15.00	15.1	
Recovery spike 1 [%]	[90 - 110]	104±7	Verified
Recovery spike 2 [%]	[90 - 110]	95±6	Verified
Repetability (CV)			
EBC non spiked	< LOD	n.d.	
EBC spiked 1 (5 pg/ml)	20%	11.1	Verified
EBC spiked 2 (15 pg/ml)	10%	5.9	Verified
Accuracy			
Reference value EBC spiked 1 [pg/ml]	5.00		
u_{ref}	2.0		
Maximum accepted difference	50%		
Normalized range	2	0.38	Verified
Maximum accepted range [pg/ml]	7.50	7.34	Verified
Minimum accepted range [pg/ml]	2.50	4.23	Verified
Reference value EBC spiked 2 [pg/ml]	15.00		
u_{ref}	2.0		
Maximum accepted difference	15%		
Normalized range	2	0.04	Verified
Maximum accepted range [pg/ml]	17.25	16.87	Verified
Minimum accepted range [pg/ml]	12.75	13.29	Verified

Concentrations measured in EBS samples collected in the field study

We did not detect 8-OHdG nor 8-isoprostane EBC obtained from the healthy volunteers in our laboratory or from the 26 workers (Table 17).

Table 17. *Results obtained for the calibration, QC and EBC samples*

	CALIBRATION CURVE				QC 5 (pg/ml)		AVERAGE EBC CONCENTRATION
8-OHdG	Slope	Intercept	R^2	LOD	Theoretical	Experimental	
Healthy (n=10)	0.032	-0.0019	0.999	0.5	5	4.86 ±0.11 (n=4)	<LOD
Asthma (n=7)							<LOD
COPD (n=9)							<LOD
8-isoprostane							
Healthy (n=10)	0.065	0.034	0.996	1.0	5	5.06±1.06 (n=4)	<LOD
Asthma (n=7)							<LOD
COPD (n=9)							<LOD

n=the number of measures

II. Optimize and characterize the determination of oxidative potential in the exhaled air - OPEA

1. Context

The development of a sensing system for determining the oxidative potential directly in the exhaled air provides non-invasive and rapid access to biochemical information related to oxidative stress and inflammation in the deep lung. Chemically, the oxidative potential describes the redox balance status of the analyzed aerosol – here the exhaled air – versus Fe(III) / Fe(II) taken as reference couple. The presence of both oxidizing molecules (e.g., H_2O_2 and ROS) and antioxidants (e.g., ascorbic acid, glutathione) in the exhaled breath is quantitatively reflected in the corresponding OPEA value.

A first version of the analyzer for oxidative potential in the exhaled air (OPEA) was developed in our group in 2016 in the frame of the OxAirdirect project funded by ANSES (French Agency for Food, Environmental and Occupational Health & Safety). The OPEA analyzer was tested in collaboration with the Service pathologies professionnelles et de l'environnement (Créteil, France) via a single-center and observational pilot study in COPD patients and healthy subjects from construction industry. This study was published in 2022 in **Medicine in novel technology and devices** [215]. Nevertheless, this system that relied on a one-step OPEA determination scenario in which the air sample directly bubbled into the reaction medium presented some drawbacks that had to be solved in the next OPEA analyzer generation: the difficulty to get rid of residual reactive reagent (FOX) in the air injection needle and the turbulences during bubbling generating projections of microdroplets that interfere with the signal. I was in charge of several optimization strategies in the frame of my PhD project to avoid these contamination issues and their suitability were evaluated.

2. Methods

Direct determination of the OPEA as a biological indicator of the redox balance status in airways requires an extremely sensitive sensing approach to counterbalance the high dilution of the exhaled air matrix. The mode of detection used in the OPEA analyzer is based on a physical principle - multiscattering-enhanced absorbance – developed by Suarez et al., [247-249] that allows the efficient conversion of any colorimetric test into a more sensitive photonic sensor compared to standard colorimetric assays based on Beer-Lambert Law (Figure 31).

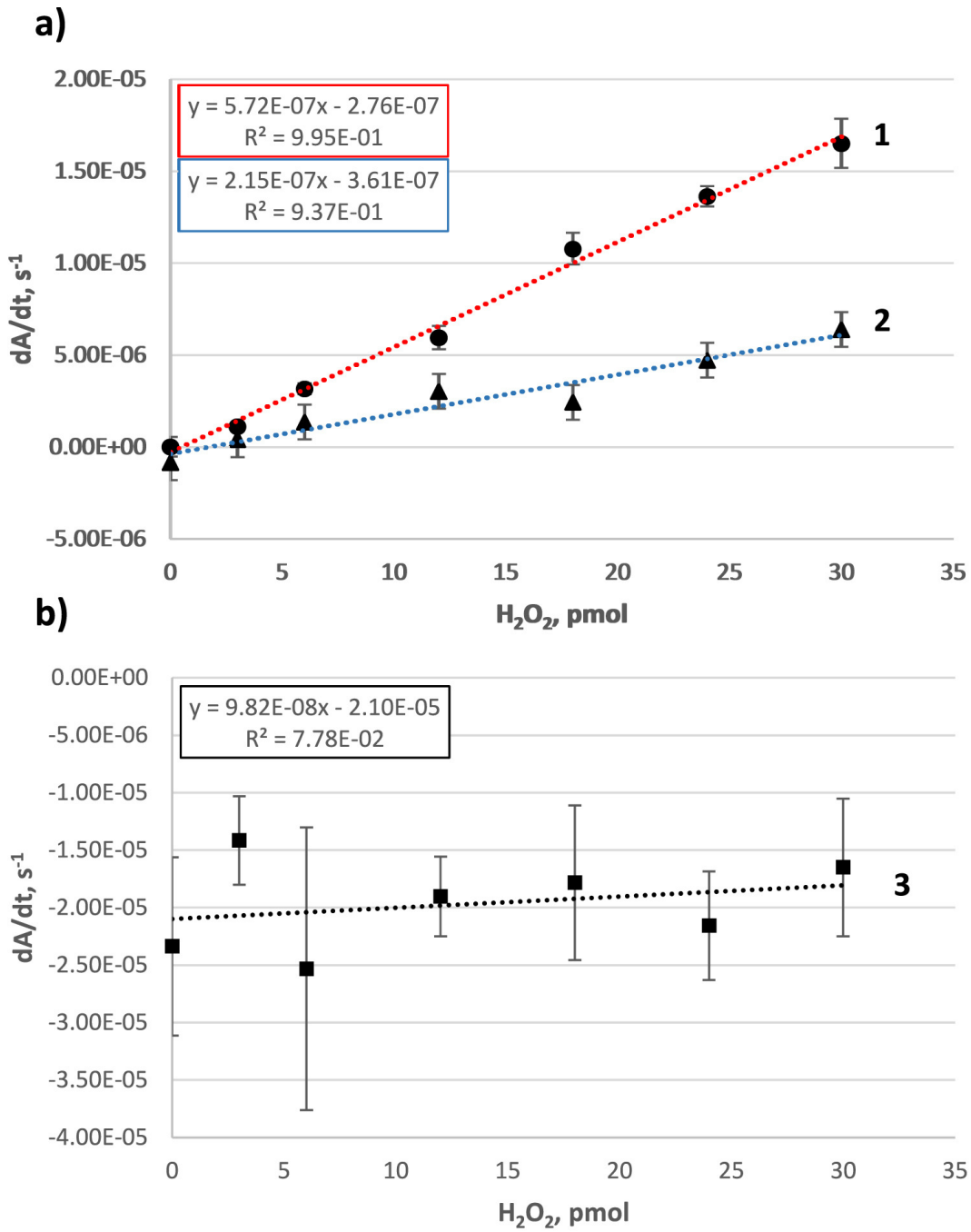


Figure 31. *Efficiency of multi-scattering amplification of absorbance strategy compared to standard colorimetric assays based on Beer-Lambert Law on analytical performances of the FOX assay at low H_2O_2 range a) calibration curves based on multi-scattering amplification and internal-reflection b) calibration curves based on Beer-Lambert absorption performed with UV-Spectrophotometer.*

Concretely, when light undergoes multiscattering through an optically chaotic medium - defined by a spatial heterogeneity of the refractive index of the medium - its optical mean path is significantly lengthened, resulting in a corresponding increase in the measured absorbance. In the case of OPEA determination, the sensing strategy consisted of coupling the colorimetric assay called FOX (Fe-Orange Xylenol) to a photonic cell enabling multiscattering to occur resulting in a strong enhancement of the analytical performances (i.e. limit-of-detection, sensitivity, dynamic range). The FOX assay relies on the presence of Fe(II) in highly acidic medium, that gets oxidized into Fe(III) in the presence of oxidizing molecules in the sample. Then, Fe(III) is chelated with orange xylenol to form a complex that strongly absorbs light at 580 nm. The presence of sorbitol acts as a chemical amplifier, which contributes to lower the limit-of-detection. Conversely, the presence of antioxidants in the sample breaks down the absorbing complex via the reverse reaction resulting in the decrease of the measured absorbance.

Since its first version, major modifications have been made to the OPEA detection system in order to: i) reduce optical interferences due to the presence of micro-sized bubbles adding a reference light source in the near-infra red; ii) increase the volume of exhaled air analyzed while reducing the analysis time; iii) automate the measurements and reduce the manufacturing cost.

The current optical elements rely on cheap optoelectronic components: orange (measurement) and near-infra red (reference) LEDs (3 mm) for light sources and RGB CMOS sensors to efficiently count the photons (unit: µseconds) giving rise to a compact and low-cost measurement system. Upstream to analysis, the OPEA analyzer is equipped with an air membrane pump to efficiently interface the air sample with the collection medium – primarily H_2O and finally D_2O – that is subsequently analyzed.

a. Procedure

The FOX reaction medium is packaged in disposable 700 µL vials and stored at -20°C before use. The current signal amplification mode is based on the use of a Teflon diffusing insert held in an aluminum reflecting cavity. The light source - an orange LED whose emission spectrum is superimposed on the absorption spectrum of the Fe (III)-OX complex - diffuses through the Teflon and propagates within the reflecting cavity where the colored reaction medium is located. The elongation of the optical path in the enclosure results in an amplification of the measured absorbance. The design of the cell allows the analysis of the entire reaction volume.

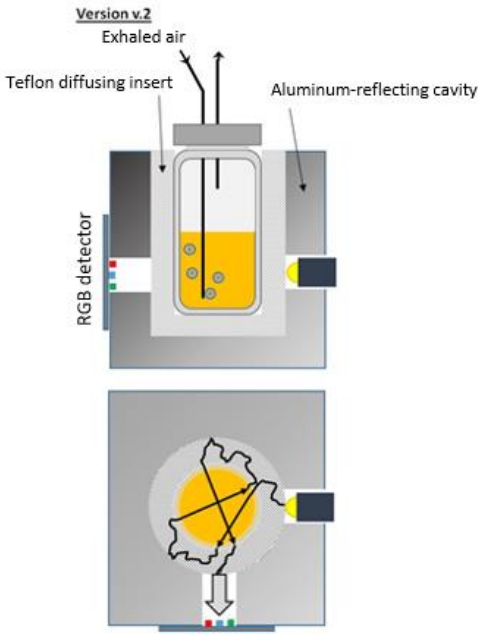


Figure 32. *Schematic representation of the first-generation photonic system - without instrumentation - for measuring the oxidizing potential on an external amplification mode in which a Teflon diffusing insert inserted in an aluminum-reflecting cavity allows an increase in sensitivity and the analysis of the total volume of the reaction medium.*

The current version is equipped with an acquisition, control and communication interface based on an Arduino-UNO module (Arduino, Italy). Changes in the absorption spectrum due to the interaction of the FOX reaction medium with the air sample after bubbling are detected by an integrated circuit (TCS230, TAOS, US) which contains an array of four photodiodes. The integrated circuit converts the irradiance of the different spectral bands - red, green, blue and total - into a digital signal allowing serial communication interfacing with the Arduino module. The spectral data are measured continuously over a period of 2 seconds and are stored on a computer (USB interface) for analysis by a generic digital program.

b. Method of collecting exhaled air

The method of collecting the alveolar part of the exhaled air: the volunteer breathes normally in a disposable mouthpiece connected to a Medivac Capnometer Valve (AAV14), which allows the alveolar and bronchial fractions to be separated automatically for each breath. In the method, the alveolar fraction is recovered in a TEDLAR bag (CELscientific corp., Cerritos, USA) with a capacity of 1 L. The absorption effect is minimal and the bag can be stored up to

15 min before measurement. The effect of light on the measurement is not significant. The design of the optical cell and the overall box considerably minimize any light interference originating from the surrounding environment. Additionally, previous experiments conducted at Unisanté have shown that the measurement method is more sensitive when applied to the alveolar fraction of the exhaled air. Other studies have also shown an increase in the concentration of droplets of fluid secreted by the respiratory epithelium in the alveolar portion of exhaled air [184]. The contribution of the bronchial fraction to the concentration of certain markers of oxidative stress - including H_2O_2 - is significant. The use of alveolar air in the proposed procedure also reduces contamination problems (environment, mouth...), allows more accurate measurements and better comparability between subjects [250].

Figure 33. *Exhaled air sampling system comprising the disposable mouthpiece (1) connected to a capnometer valve (2) which allows the alveolar fraction of the sample to be recovered in a 1L Tedlar bag*

c. Analysis of the sample

Immediately after the exhaled air sampling, the sampling bag is connected to the OPEA analyzer system containing a bubbling cell with 1mL of water (initial measurements) or deuterium oxide (optimized measurements). The "on" control of the software is activated to trigger the bubbling of the sample in water for 4 min at a flow rate of 250 mL/min. After bubbling, 2*150 µl of aqueous air collection sample is withdrawn from the collection medium and injected into the FOX solution (700 µL). The reaction medium (consumable) is then placed on the instrument, and the optical cell starts the absorption measurement ($\lambda_{580}/\lambda_{NIR}$) over a period of 3 min with a time resolution of 2 s. The analysis consists of calculating the slope corresponding to the variation of the signal measured by the photon sensor in the measurement interval Δt (s) = t_{150}-t_{60}, t_0 corresponding to the end of the sample bubble sequence. Each slope value (s^{-1}) is converted into equivalent of H_2O_2 (pmol/L_{air}) through calibration procedures.

In order to take into account the contribution of oxidizing agents present in the ambient air inhaled during the exhaled air collection phase, the OPEA is calculated as the difference between the oxidative potential of the exhaled air and the oxidative potential of the ambient air: OPEA (pmol/L_{air}) = OP_{exh} – OP_{amb}

Prior to measurements the OPEA analyzer is let to warm up for 30 min in order to stabilize the emission spectra of the LEDs.

The sequence of the test is carried out in four consecutive operations, as follows:

Sampling of 1 L of exhaled air

⇩ 1min

Air collection via bubbling in 1ml of D_2O

⇩ 4min

Injection of 300 µl of collection D_2O vial into FOX

Acquisition of measurement data

⇩ 3min

Data Analysis

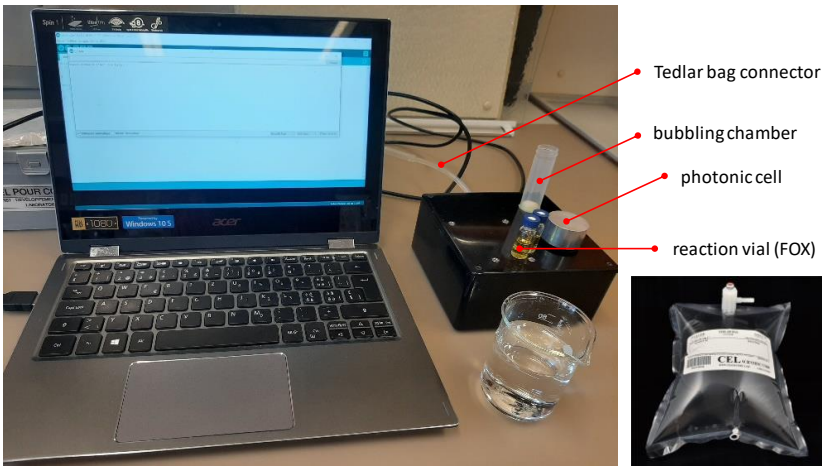


Figure 34. *Descriptive picture of OPEA analyzer controlled with Arduino software. The air sample bubbling and the OPEA analysis occurs in two consecutive steps. EA sample from the volunteer is directly collected in the TEDLAR bag (bottom right) and plugged to the OPEA*

system for analysis. The air sample is forced to bubble in D_2O (1 mL) during 4 min (250 mL/min). Then, the analysis is performed by introducing 300 µL of the collection solution into the reaction vial containing 700 µL of FOX prior to the photonic measurements (3 min). Triplicate measurements can be made per air sample.

3. Experimental optimization of the OPEA measurement

a. Calibration of OPEA analyzer with hydrogen peroxide

The OPEA analyzer system was calibrated with standard solutions of hydrogen peroxide as standard ROS and also considered as the main composite of OPEA, with a half-life of 10^4 seconds (about 2.45 hours) in a concentration range between 0 and 1 µM. The addition of 100 µl of standard solution to the reaction vial allows the establishment of a calibration curve and the determination of the detection limit. The linear relationships between hydrogen peroxide and the response for the OPEA analyzer are described by the following equations: 10^5.slope = 0.0213.[H_2O_2] + 1.473 (OPEA analyzer). The limit of detection was calculated as 3σ for the blank and was particularly low, with a value of is about 10 pmol/L_{air} when H_2O is used as the air collection medium. Importantly, the relative variability of the blank was attributed to the possible presence of ROS in the ultrapure water used. Milli-Q water stands for ultra-low conductivity and treated with UV-C as germicide process, that is known as a potential source of ROS generation. In order to circumvent this issue and further improve the limit-of-detection of the OPEA analyzer, the H_2O was replaced by D_2O that shows stronger stability as a blank.

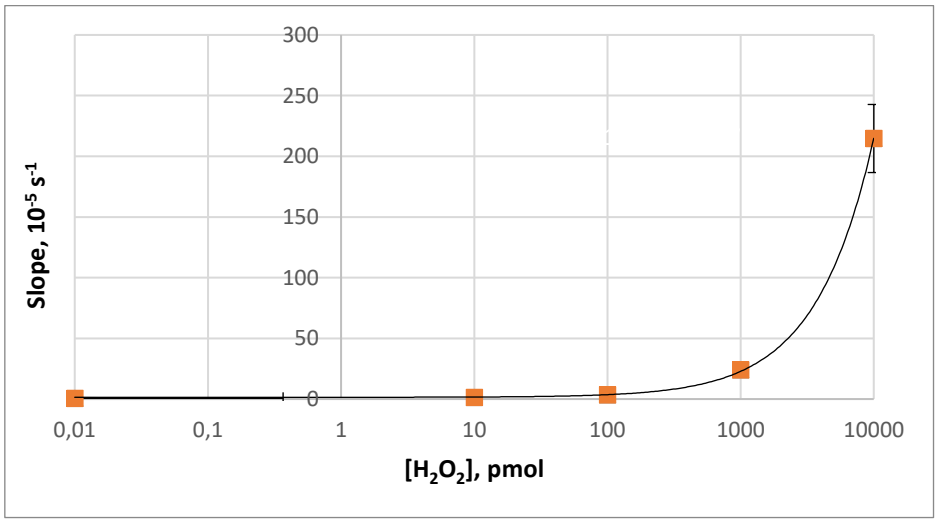


Figure 35. *Calibration curve obtained with OPEA analyzer with [H_2O_2] ranging from 0 to 1 µmol in 1 mL of FOX reactive reagent. For readability, only the curve fraction from 0 to 10 nmol is*

shown in logarithmic scale. For each [H_2O_2] triplicates were used and the corresponding error bars are shown on the curve

b. Optimization of OPEA sampling and measurement conditions

Quantity of exhaled air sample volume:

When the volume of exhaled air analyzed is limited to 150 mL, the corresponding measurements with healthy volunteers are in the order of magnitude of the detection limit. The improvement made was to increase the sample volume to 1 L of air and the bubble rate from 50 to 250 mL/min. In this way, the OP values measured were significantly higher than the LOD.

Stabilization of the reaction medium prior to measurements:

The vials containing the reaction medium – namely FOX solution – are stored at -20°C in the dark and the blank measurements carried out directly after thawing give slope values that are much higher than the usual blank values (cf. calibration) and disturb the reproducibility of the measurements. The stabilization step consisted of shaking the contents of the vial for a few seconds (vortex) in order to make the dissolved molecular oxygen react with the reaction medium (FOX). This process resulted in highly reproducible OPEA measurements (standard deviation: 5%; n= 5). Anti-foam was also tested in reaction medium formulation to stabilize the solution before measurement. However, this last one was quickly withdrawn because of due to chemical interferences with FOX assay.

Reduction of contamination:

Optimization phases were carried towards the most repeatable reaction by reducing exogeneous chemical contaminants. Figure 36 presents the values of the blank when different collection medium are used: Milli-Q water vs deuterium oxide (D_2O). Also, the effect of HNO_3 treatment on the vials – in order to remove the metals having a pro-oxidant behavior – was evaluated. D_2O and HNO_3 treatment appeared to be the most appropriate conditions for FOX analysis showing a significant lower variation in the blank response ($p<0.05$). Milli-Q Water plays a significant role in the contamination because it already contains ROS that can react this the FOX reaction medium. These ROS are produced during the initial purification which consists of a passage of water through an ion-exchange resin system providing a variable contamination and the photo-oxidation by UV-C lamp which conducts to ROS generation.

Therefore, in the course of the study it was decided to substitute D_2O to Milli-Q water and systematically use HNO_3 treatment for the glass vials.

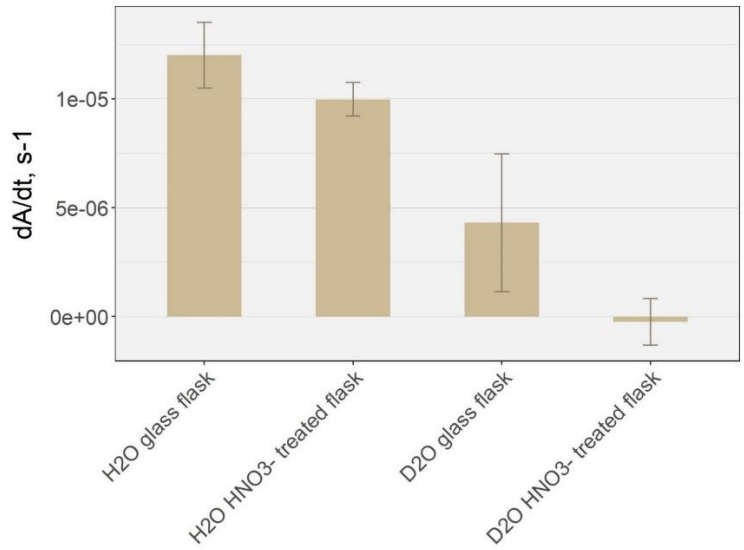


Figure 36. *Contribution of the water type on the slope in blanks samples. The error bars correspond to the standard deviation for a minimum of three measurements*

Optimization of instrumentation:

a) An internal reference was added in the system by the use of a near-infrared light source (750 nm) which emission wavelength does not overlap with the absorption spectrum of the Fe (III)-OX complex. Alternate yellow/near-IR illumination of the optical cell enabled to reduce considerably the optical interferences due to micro-bubbles generated into the reaction vial during the injection of liquid from bubbling cell into the vial containing FOX.

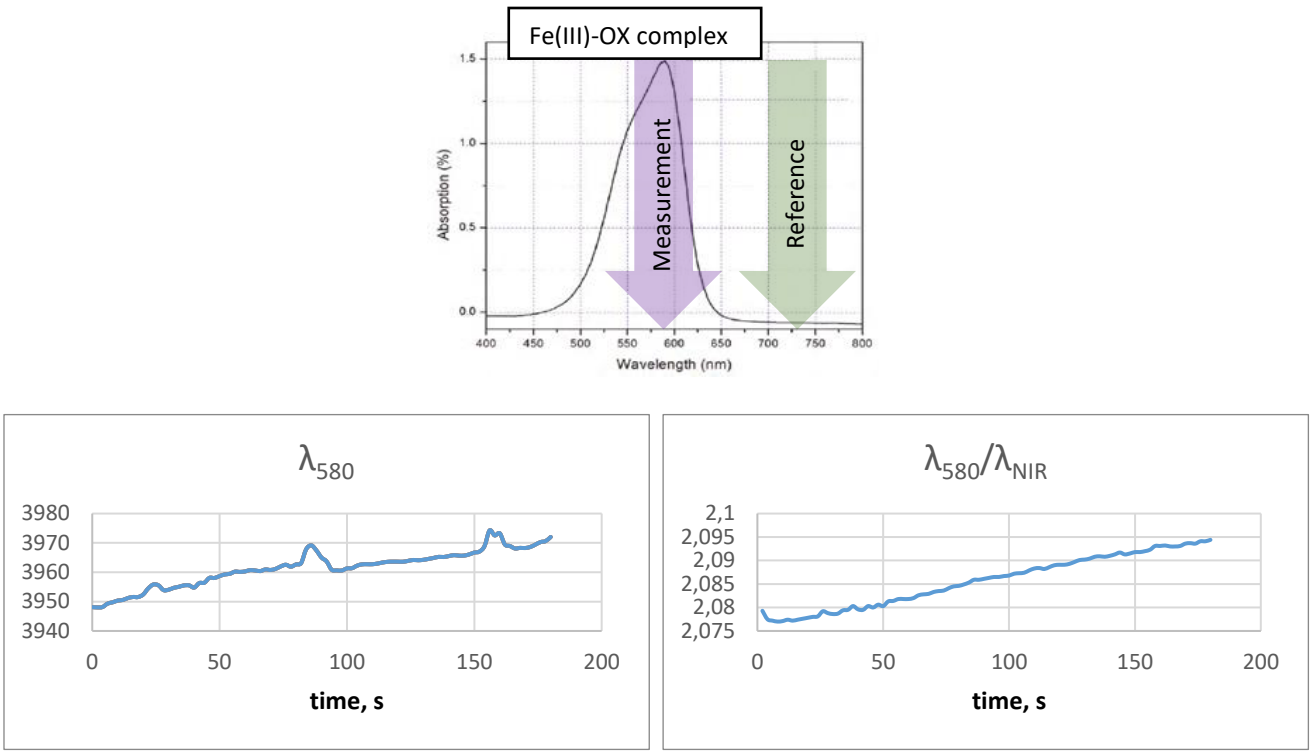


Figure 37. *Reduction of optical interferences by the use of a near-infrared (750 nm) light source as normalizing signal*

b) A new device with three channels with the capability of measuring three replica simultaneously has been developed and used during the field campaigns (R*efer part 4*). This improvement allowed us to significantly increase the throughput of the OPEA measurements. To validate the device, each channel was calibrated separately and a factor of correction was applied to each channel according to its own sensitivity. The device is presented in the Figure 38.

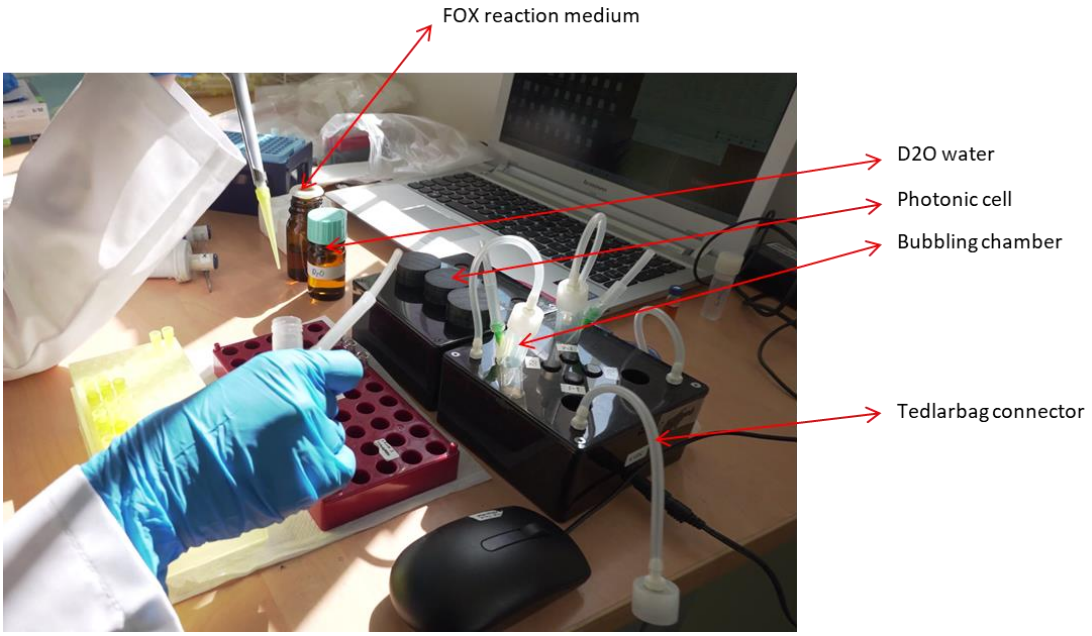
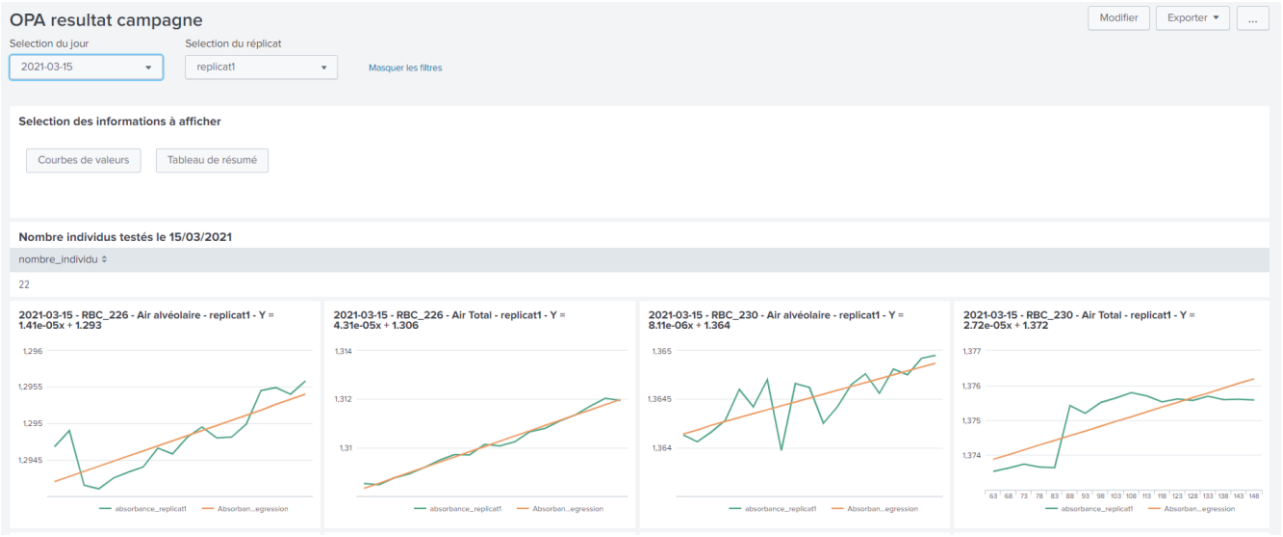


Figure 38. *Descriptive picture of OPEA analyzer with three measurement channels controlled with Arduino software utilized during field campaign*

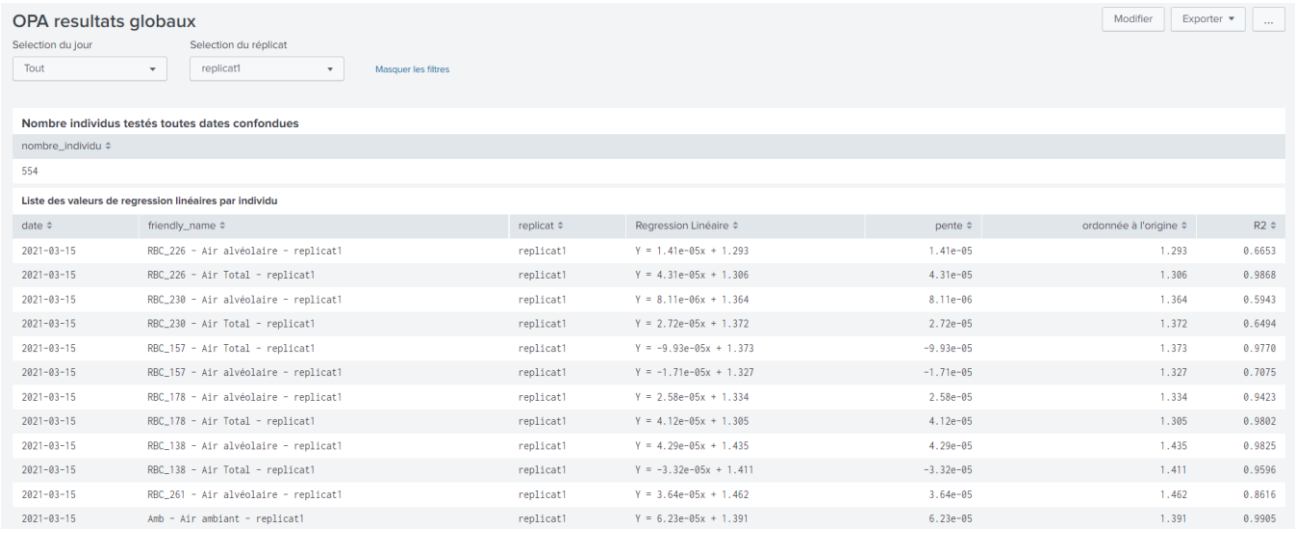
c. Computing optimization of data analysis

Until recently, the data was processed manually on Excel by calculating the slopes measurement interval Δt (s) = t_{150}-t_{60}. At the beginning of the field campaigns, where a larger number of volunteers was expected (300 people), the manual processing of the raw data appeared a highly time-consumming step and a potential source of errors. The exploitation of the measurement data would have required more than 3 months to draw the linear regression curves of the raw absorbance data over time. In this context, a data treatment solution was found with **Spunk® software** which use remains free providing the relatively low flow of data treated. The measurement data of the OPEA method are log files in text format with a size of a maximum of 3 MB per campaign; much less than the maximum authorized of 1 GB to maintain a free license. Splunk classifies in real-time the data from 'log' format obtained by the Arduino software in graphical reports as well in the form of interactive dashboards (as represented in the Figure 39) During my PhD, I, contributed together with a computer scientist to develop the Splunk solution for OPEA data analysis.

a)



b)



date	friendly_name	replicat	Regression Linéaire	pente	ordonnée à l'origine	R2
2021-03-15	RBC_226 - Air alvéolaire - replicat1	replicat1	Y = 1.41e-05x + 1.293	1.41e-05	1.293	0.6653
2021-03-15	RBC_226 - Air Total - replicat1	replicat1	Y = 4.31e-05x + 1.306	4.31e-05	1.306	0.9868
2021-03-15	RBC_230 - Air alvéolaire - replicat1	replicat1	Y = 8.11e-06x + 1.364	8.11e-06	1.364	0.5943
2021-03-15	RBC_230 - Air Total - replicat1	replicat1	Y = 2.72e-05x + 1.372	2.72e-05	1.372	0.6494
2021-03-15	RBC_157 - Air Total - replicat1	replicat1	Y = -9.93e-05x + 1.373	-9.93e-05	1.373	0.9770
2021-03-15	RBC_157 - Air alvéolaire - replicat1	replicat1	Y = -1.71e-05x + 1.327	-1.71e-05	1.327	0.7075
2021-03-15	RBC_178 - Air alvéolaire - replicat1	replicat1	Y = 2.58e-05x + 1.334	2.58e-05	1.334	0.9423
2021-03-15	RBC_178 - Air Total - replicat1	replicat1	Y = 4.12e-05x + 1.305	4.12e-05	1.305	0.9802
2021-03-15	RBC_138 - Air alvéolaire - replicat1	replicat1	Y = 4.29e-05x + 1.435	4.29e-05	1.435	0.9825
2021-03-15	RBC_138 - Air Total - replicat1	replicat1	Y = -3.32e-05x + 1.411	-3.32e-05	1.411	0.9596
2021-03-15	RBC_261 - Air alvéolaire - replicat1	replicat1	Y = 3.64e-05x + 1.462	3.64e-05	1.462	0.8616
2021-03-15	Amb - Air ambiant - replicat1	replicat1	Y = 6.23e-05x + 1.391	6.23e-05	1.391	0.9905

Figure 39. *Example of Splunk a) graphical reports b) OPEA dashboard. Possibility to select the day and the measurement replicate (1 to 3), indication of the number of individuals measured during the day for each measure (denoted by an id) is associated a slope, an ordinate and a regression line.*

d. OPEA characterization sub-study

With the approval of the Swissethics Committee (CERVD HRO 2018-00986). A study is currently on-going which aim is to determine the inter-/intra-individual variability of OPEA measurements with control (n=5) and COPD (n=5) volunteers. This sub-study of the present work (the sampling protocol described in Figure 40) should help defining the origin of the

different exhaled ROS in the lung (proximal vs. distal) for healthy and COPD participants; the within-subject variability over time on healthy volunteers; the influence of potential exogenous interfering compounds on the selected analytes; the optimal conditions for the analytical quantification of OPEA and standardize the procedures in order to make them usable for a wide range of populations.

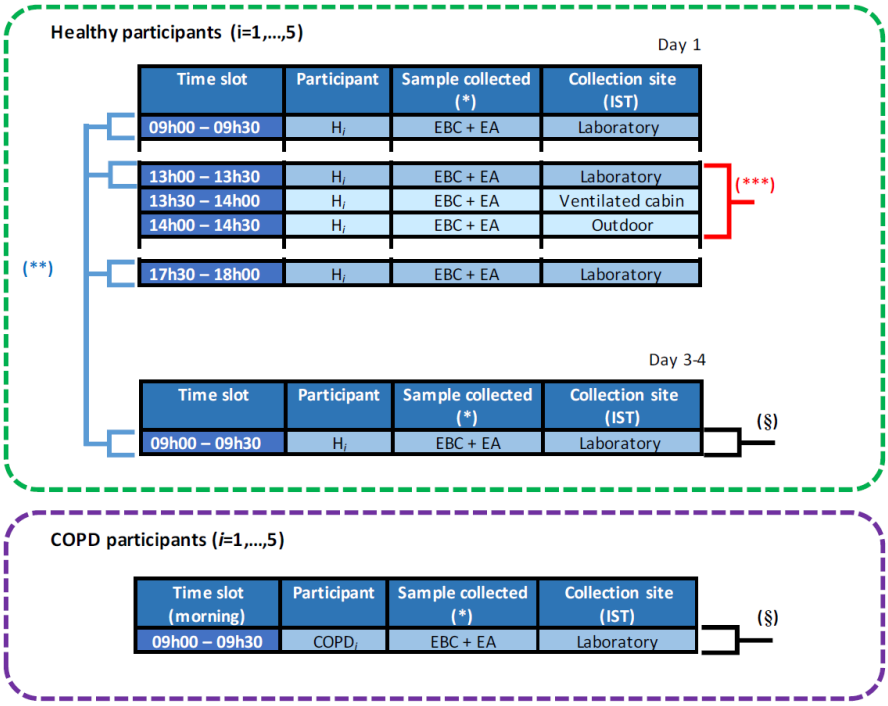


Figure 40. *Schematic representation of the procedure followed by sub-study indicating the time points for EBC and EA collection on two groups of participants: healthy (n=5) and COPD (n=5) volunteers*

So far, only healthy control volunteers have been studied in our laboratory (n=5) and we still seek for recruiting COPD volunteer through the pneumology service of CHUV. This difficulty of recruitment has to be linked with the general context of the Covid-19 pandemic where COPD patients are considered as high-risk group.

The five healthy volunteers were recruited within a period of 13 months (08/2019 to 09/2020); the OPEA analyses were performed at Unisanté laboratory.

Table 18. *OPEA (alveolar (OPEA_AA) and total air fractions (OPEA_AT) and ambient OP values calculated for healthy controls (n=5) at different sampling time and under different environments, as described in the protocol of Figure 39. All OPEA and OP ambient values are expressed in pmol/L_{air}*

Subject ID		t_1	t_2	t_3	t_4	t_5	t_6	Mean	SDEV_intra
1	**OPEA_AA**	-7	-7	2	n.a.	n.a.	-2	-3,6	4,3
	OPEA_AT	6	-4	-2	-4	3	n.a.	-0,2	4,6
	Ambient	40,2	41,8	41,3	41,3	35,3	39	39,8	2,4
2	**OPEA_AA**	0,9	7,5	14,9	34,5	27,6	9,4	15,8	12,8
	OPEA_AT	-4,4	11,0	1,4	13,3	n.a.	42,5	12,8	18,1
	Ambient	32,9	34,0	32,0	41,1	34,0	35,6	34,9	3,2
3	**OPEA_AA**		-25,4	-14,0	-116,7	-30,9	-12,8	-20,8	8,8
	OPEA_AT	-11,2	-10,6	-40,4	-129,3	-19,6	-4,5	-17,3	14,0
	Ambient	81,6	77,9	73,4	145,0	57,1	19,8	62,0	25,4
4	**OPEA_AA**	-17,6	-20,9	-2,7	-54,6	-17,1	-15,4	-17,8	2,3
	OPEA_AT	-12,3	3,8	-0,3	-51,4	-19,7	-15,9	-11,0	10,3
	Ambient	28,0	34,5	20,0	75,5	46,2	25,5	33,6	9,2
5	**OPEA_AA**	n.a.	-5,3	-5,0	-6,9	-5,8	-2,8	-4,7	1,6
	OPEA_AT	n.a.	-7,1	-5,0	-28,4	2,3	1,8	-1,0	5,3
	Ambient	23,7	29,1	12,4	41,0	13,7	11,9	19,6	8,2

From the OPEA values obtained with the five volunteers at the different time-points of the day, it is noticeable that only one volunteer – subject_2 – shows positive values. As expected from earlier study carried out with both COPD and healthy controls [215], positive OPEA value (OP_{exh}>OP_{amb}) would be indicative of the COPD status probably due to the inflammatory context and the presence of oxidants originating from the lung lining fluid (or depletion of antioxidants). Therefore, OPEA data from volunteer_2 (mean values of 16 and 13 pmol/L_{air} for OPEA_AA and OPEA_AT, respectively) cannot be considered as control reference values. Statistical analysis of OPEA for healthy controls (n=4) indicate that OPEA_AA values result lower than corresponding OPEA_AT ones, although the difference is not significant (t-test; p> 0.14). The standard deviation calculated for the overall OPEA values is of 9 and 11 pmol/L_{air} for OPEA_AA and OPEA_AT, respectively.

As summarized in

Table 18, the intra-subject variability along the day and at day+3 was evaluated for each volunteer and results show some disparities: for volunteer_1, 4 and 5 standard deviation is low in comparison to the inter-subject's one, whereas volunteer_2 and 3 exhibit standard deviation much higher values, up to 18 pmol/L_{air}. In the case of volunteer_2, the fact that OPEA is positive might be indicative of an inflammatory process taking place, but no further deduction should be rationally done at this stage. For volunteer_3, it is noteworthy that the corresponding values for ambient OP are more widely distributed with a standard deviation of 25 pmol/L_{air} versus 2, 3, 8 and 9 pmol/L_{air} for the other volunteers. Finally, the experiments performed under higher ambient OP (outdoor conditions) enabled reaching significantly higher OP values – more than two-folds – than for indoor conditions for volunteers_3, 4 and 5. In most of those cases (5/6) the corresponding OPEA value results strongly affected and drop down to significantly lower range. Thus, during field campaign measurements the level of ambient OP should be regularly determined along the day in order to keep the resulting OPEA values in a comparative frame.

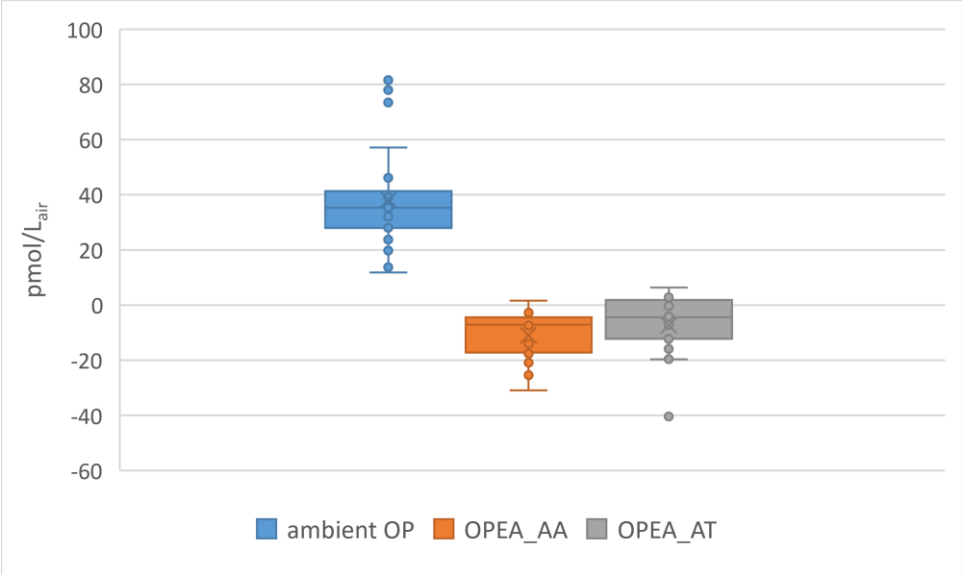


Figure 41. *Box-plot representation of overall OPEA values (n=18; n=19) obtained for the four volunteers with low OPEA values as expected for healthy controls (values corresponding to volunteer_2 are excluded). The ambient OP values correspond to indoor environments (laboratory) measured at the different time-points of the study. OPEA_AA = alveolar fraction of OPEA, OPEA_AT= total fraction of OPEA.*

FEASIBILITY OF SELECTED OXIDATIVE STRESS BIOMARKERS IN A PILOT FIELD

I. Context

1. Contribution to this part

I provided all tables, wrote the protocol for the pilot study submitted to the ethics committee, which served as the basis for the article published in Journal of Occupational Medicine and Toxicology with the heading "Respiratory Disease Occupational Biomonitoring Collaborative Project (ROBoCoP): A longitudinal pilot study and implementation research in the Parisian transport company". I actively contributed to the research plan development with respect to my research domains: chemistry and pharmacology.

2. Aims and design of the studies

The project encompasses two consecutive studies consisting of a **pilot study** followed by a **field study**. The studies were conducted in the Parisian transport company (45,000 active workers), and particularly its underground subway, as subway indoor air was reported the most PM polluted urban transport environment [251-255].

The **pilot study** is a longitudinal exposure assessment and biomarker study, serving as an orientation to the field study. This pilot study aims to:

1- Understand the suitability of the candidate biomarkers in surveying populations at risk such as workers exposed to COPD causing agents through a description of relationships between parameters characterizing exposure, exposure biomarkers and effect biomarkers.

2-Better estimate the exposure related specifically to each of the occupations in the EFS through the use of determinants and integrative exposure measurements

3- Determine the best sampling plan with respect to the half-life of the candidate biomarkers;
4- Implement and validate the sampling procedures and analytical methods;

5- Select the best suitable biomarkers to be then measured in the field study.

The pilot study is planned to follow each study participant daily during the work-shift for two consecutive weeks.

The **field study** has an implementation research design. Within the context of field trials, implementation research focuses on "optimizing the delivery of existing interventions that have previously been shown to be efficacious when implemented well" [256]. In our context, the field study will enable us to demonstrate the applicability of the standardized protocol for biomarker measurements in occupational settings while assessing the biomarkers validity.

II. Materials and methods

1. Research setting and participants

The research was conducted in a Parisian urban transport company in France for the experimental part, at RATP Laboratory of Essays and Measurements (LEM) (only pilot study) and at Unisanté in Switzerland for the analytical part. The study samples comprised three categories of underground subway workers having their workstations underground: locomotive operators, security guards, and station agents in charge of information, ticket sale and control. Station agents oversee passenger information and ticket sale. They had both a fixed workstation, operating the ticket counters, and mobile activity. The mobile activity included intervening on ticket distributors in station concourses and information and ticket control rounds all along the subway lines of their assignment. Locomotive operators conducted the subway trains and spent most of their work-shift inside the train cabin. Security guards constantly moved from station to station on demand and in contrast with two other types of professionals had no particular subway line assigned. These jobs are considered the most exposed to PM compared to other professionals working outdoor, such as bus or tram drivers, controllers and administrative staff. They are also less concerned with other chemical exposure compared to maintenance workers, which could bias the study.

The **pilot longitudinal study** used a convenience sample of nine workers- three workers per occupation. Both women and men aged 40+, and non-smokers for at least 10 years (to avoid interference of smoking with the exposure-biomarkers-health outcome relationships) were considered eligible. The only exclusion criterion was a counter-indication of spirometry test and acute or chronic morbidities other than COPD. The participants had their workstations on subway line 7. This line is entirely underground, deep, has no mechanical ventilation and

therefore represents one of the worst-case scenarios in terms of exposure and one of the best for setting a pilot study. It offers a possibility to set up rooms for participant reception, biological sampling and equipment storage. The workers recruitment was managed by the medical coordinator of the study. The medical coordinator organized internal company meetings with workers, their supervisors and occupational physicians. At these meetings, workers were informed on the objectives of the pilot study, inclusion criteria and invited to contact their occupational physician to declare their wish to participate in the study and to arrange an individual interview for this purpose. The occupational physicians checked the inclusion and exclusion criteria and collect the signed written informed consent form eligible workers. The recruitment of workers was carried out in chronological order: The eligible workers who volunteer first were given priority.

The data collection lasted six weeks, from 7 October 2019 to 15 November 2019, and was organized by job type: two weeks per type of subway professionals.

For the **field study,** we included both male and female adults regardless their smoking status. The only exclusion criterion was a counter-indication of spirometry test (e.g., recent surgery to the head, chest, stomach, or eye, unstable angina, excessive hypertension, or a recent myocardial or stroke). We constructed a probability sample maximizing the number of workers at risk with regard to the primary health outcome (COPD). For this, an automatic stratified randomization procedure was applied on the register of 10,778 underground workers prepared by the company's human resources department. The strata were defined by four variables: sex, age, smoking status (smokers, ex-smokers, non-smokers) and exposure (depending on the occupation: station agents, security guards and drivers). This sample had to comprise at least 300 workers who would provide a signed written informed consent for participation, answer the epidemiological questionnaire and participated in the medical check and biological sample collection, as described further.

The sample size was calculated assuming a 13.7% prevalence of COPD [257] and the statistical distribution of the OPEA values corresponding to the preliminary study conducted in a clinical setting [258]. The sample of 300 participants allow discriminating with 90% power COPD cases from the controls with an area under the ROC curve (AUC) of 0.64 or 0.70. These values correspond to an absolute or a relative difference between OPEA and the oxidative potential of the indoor air, respectively. If including 400 subjects, these are respectively 0.66 and 0.72. Thus, we decided to limit our study to 300 participants.

Table 19 gives the results of these simulations.

Table 19. *Simulation of the number of participants*

	Absolute difference		Relative difference	
	mean	5th pct – 95th pct	mean	5th pct – 95th pct
Sensitivity	0.66	0.54 – 0.79	0.57	0.44-0.71
Specificity	0.8	0.75 – 0.84	0.93	0.91-0.96
Positive predictive value	0.34	0.26 – 0.42	0.57	0.44 – 0.70
Negative predictive value	0.94	0.91 – 0.96	0.93	0.91 – 0.96
AUC	0.79	0.72 - 0.86	0.84	0.77 – 0.90
AUC detected with power 90%	0.64		0.7	

Assuming a participation refusal rate of 25%, a first sample of 400 workers were contacted by their occupational physician. Eligible and voluntary workers, estimated to 300 participants approximatively, were enrolled, after providing a written consent.

2. Exposure and health outcome measures

The **pilot study** was scheduled for six consecutive weeks, to allow a two-week follow up for every occupation. Both exposure and health outcomes were measured. Three workers with the same occupation were received at Porte de la Villette station at the same time an hour before their work-shift. During this time, the participant undergone a medical check including spirometry and the biological sample collection (Figure 42).

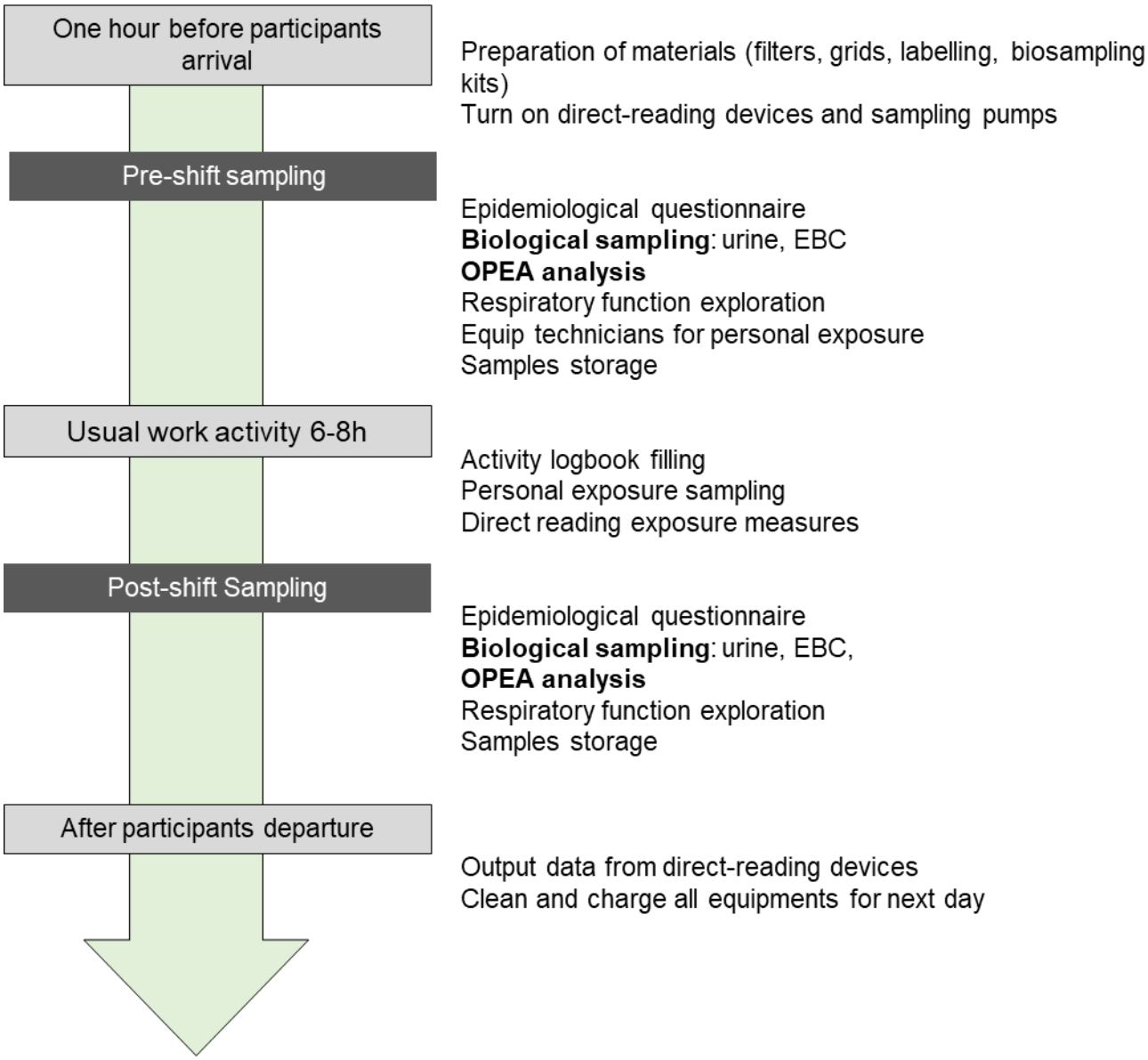


Figure 42. *Preparatory actions and measurements carried out every day for each participant during two-week prospective follow-up in the pilot study*

Figure 43. *Biological equipment at RATP sampling place for pilot study*

All workers filled in the standardized epidemiological questionnaire and daily questionnaires to provide sociodemographic, occupational, life-style (e.g., diet), and health data (e.g., medication). The latter were used to understand factors that may influence the exposure to PM and metals that might influence their biological sample analysis results. Biological sampling was planned twice a day (pre-and post-shift), while exposure to PM and other pollutants possibly present in subway were measured over the working shift. Table 20 summarizes all airborne exposure measurements, while Table 21 summarizes exposure and effect biomarkers, biological matrices and corresponding chemical analysis required.

The pre- and post-shift EBC samples were obtained following the recommendations of the American Thoracic Society and the European Respiratory Society Task Force [180], i.e. by cooling the exhaled air to -5 °C during calm oral ventilation for 2x10 minutes, using portable collection equipment (Turbo- DECCS, Medivac, Parma, Italy). None of the participants declared drinking coffee and eating within the hour before the measurement. A volume of 2 to 3 mL (means) of condensate were collected (20 minutes) and distributed in several aliquots immediately frozen at -20 °C and then stored at -80 °C (within the week) in the freezer located in the RATP's *Service Santé Travail et Prévention* (SSTP) freezer. Biological sample collection, aliquoting and storage were operated in a closed clean room equipped for study purposes.

EBC metal concentration was quantified by ICP-MS technique (ICap TQ, Thermo Scientific) at Unisanté laboratory. The calibration curve was prepared by diluting a multi-element certified stock solution (Plasma Cal, SCP Science, France) with water to 0-50 µg/L range. The HNO_3 (40 µl, Plasma Plus pure, 67-70%, SCP Science, France) containing the internal standards Y,

Rh, and Ir (100 µg/L) was added to 400 µl of calibration or EBC sample. The mixture was directly introduced in the plasma by aspiration. The QTegra vers 2.10 software was used for the signal acquisition and treatment. All metal concentrations were standardized per EBC volume and expressed in ng/mL. The observed limit of quantifications (LOQs) was 10.0 ng/mL for Ba, Co, Cr, Cu, Mn, Mo, Ni, Pb, V, 1 µg/L for Fe, Ti and Zn, 5.00 µg/L for Al, and 20.00 ng/mL for Si.

The number of sub-micron particles in the EBC samples was determined by using the nanotracking analysis (NTA), which determines the size distribution profile of small particles with a diameter of approximately 40-1000 nanometers (nm) in liquid suspension [259]. About 400 µl of EBC sample was introduced into the cell of a NTA instrument (LM10 Malvern Pananalytical, Malvern, UK), which adds a laser beam to excite the particles in Brownian motion and track their movements with a camera and a software. A total of 5 videos of 60 seconds was recorded and analyzed using the NTA software (version 3.1).

MDA, 8-isoprostane and 8-OHdG concentration in EBC were quantified following validated methods described in the part 3.

EBC concentrations of anions associated to nitrosative stress (NO_2^- and NO_3^- as stable end-product of NO oxidation) or to metabolism (lactate, acetate, propionate, butyrate, formate and pyruvate) were analyzed in EBC following a validated method [260]. Briefly, 10 µl of the EBC sample was injected without any treatment into a Dionex ICS 5000+ ion chromatograph, equipped with an analytical column IonPac AS11-HC250 mm, 4 µm (ThermoFisher Scientific, Ecublens, Switzerland) and a conductivity detector. The LOD was comprised between 0.07-0.58 µM (depending on the analyte), allowed the quantification of all these anions in all samples.

Urine was collected at the beginning and at end of the shift in appropriate flasks (250ml), previously washed with nitric acid (10%). For the morning urine collection (at the beginning of the work-shift), the collection flasks were distributed to the participants the day before. In order to avoid contamination, participants were previously informed by a team member on the urine collection procedure (collection outside the "polluted" area, hand washing). Immediately after collection, the samples were aliquoted in a tube of 8ml, acidified if necessary and frozen at -20°C on site and then transferred at the end of the day to the SSTP freezers to avoid oxidative and anti-oxidative reactions that could alter their nature prior to analysis. At least six aliquots were prepared for each participant's sample. Urinary concentrations were standardized per gram of creatinine to remove the influence of urine dilution on exposure biomarkers measured

in spot samples. Urinary creatinine concentrations were measured with the Jaffe method [261]. Only urine samples with creatinine concentrations in the normal range (0.3-3 g/L) were considered.

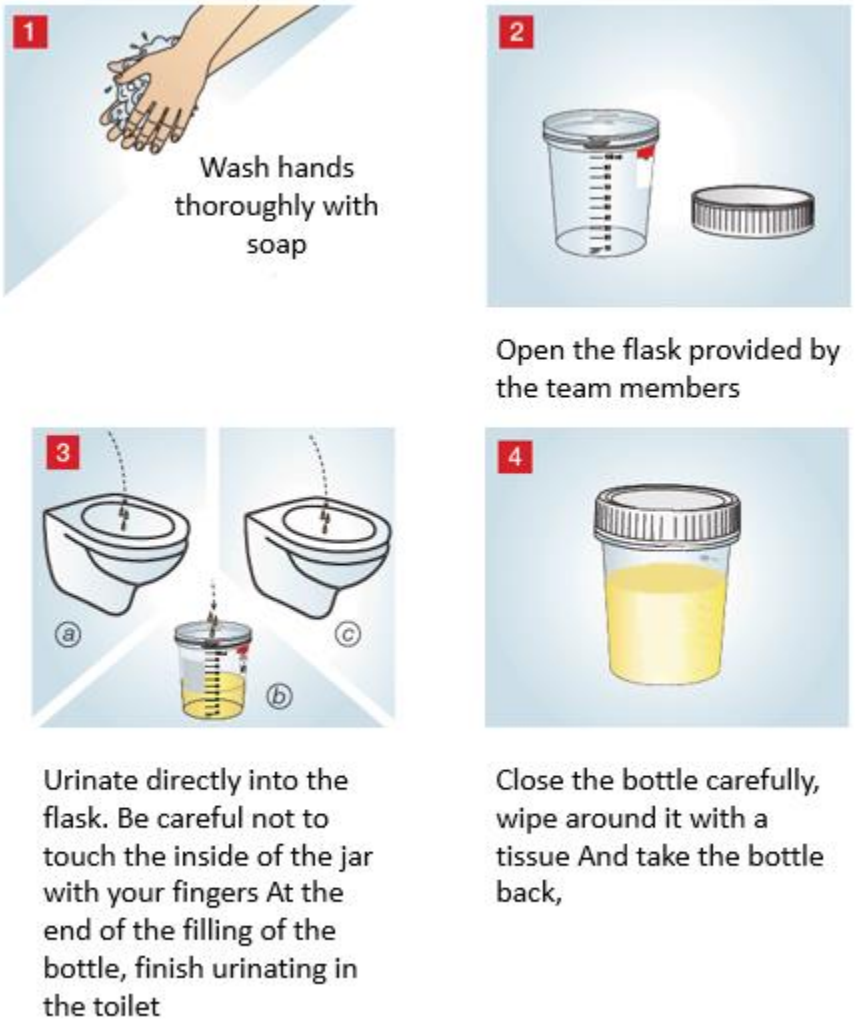


Figure 44. *Example of form for urine sampling given to the participant*

EA was sampled for the immediate assessment of OPEA. Participants were asked to breathe tidally during 1-2 minutes through a mouthpiece while wearing a nose clip, enabling collection of exhaled air into a single-use 1 L bag specifically designed for air sampling (Tedlar). This operation was repeated twice for each participant, to enable the individual collection of both the total and alveolar (distal) expired air fractions. The physical separation between both is performed by a built-in CO_2 detector valve (IR capnometer), i.e. elevated CO_2 pressure being indicative of alveolar fraction collection. In addition, one blank (H_2O), two 'quality check'

calibrations (known H_2O_2 concentrations) and two ambient air measurements per day were performed following the same measurements' procedure as for participants' total and alveolar expired air fractions.

The analysis of expired air was performed with an OPEA analyzer, immediately after sampling. The detection and quantification were based on the methodology detailed in the part 3. Triplicates measurements were performed in OPEA analyzer for each exhaled air sample. FOX reactive vials were stored at room temperature in a powder form in a closet sheltered from the light and reconstituted with the fox solvent at the beginning of each day of campaign measure. EA sample leftovers were discarded after OPEA analysis in an adequate biological material bin. All files obtained from the analysis in the Arduino software were then transferred to Splunk ® software to automatically compute the slopes associated with each measurement (Figure 45).

nom	date	weekday	file_name	ID	regression_lineaire	pente	ordonnee_origin	R2
RBC_226 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	1	Y = 1.41e-05x + 1.293	1.41e-05	1.293	0.6653
RBC_226 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	2	Y = -8.37e-06x + 2.265	-8.37e-06	2.265	0.0734
RBC_226 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	3	Y = -1.20e-04x + 2.176	-1.20e-04	2.176	0.9524
RBC_226 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	4	Y = 4.31e-05x + 1.306	4.31e-05	1.306	0.9868
RBC_226 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	5	Y = -3.34e-05x + 2.306	-3.34e-05	2.306	0.8070
RBC_226 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	6	Y = 8.13e-06x + 2.225	8.13e-06	2.225	0.3508
RBC_230 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	7	Y = 8.11e-06x + 1.364	8.11e-06	1.364	0.5943
RBC_230 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	8	Y = 4.03e-05x + 2.342	4.03e-05	2.342	0.9136
RBC_230 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	9	Y = 8.04e-06x + 2.212	8.04e-06	2.212	0.5532
RBC_230 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	10	Y = 2.72e-05x + 1.372	2.72e-05	1.372	0.6494
RBC_230 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	11	Y = -1.70e-06x + 2.387	-1.70e-06	2.387	0.0743
RBC_230 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	12	Y = 1.87e-05x + 2.155	1.87e-05	2.155	0.7878
RBC_157 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	13	Y = -9.93e-05x + 1.373	-9.93e-05	1.373	0.9770
RBC_157 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	14	Y = -5.25e-05x + 2.359	-5.25e-05	2.359	0.9354
RBC_157 - Air Total	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	15	Y = -5.22e-05x + 2.240	-5.22e-05	2.240	0.9034
RBC_157 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	16	Y = -1.71e-05x + 1.327	-1.71e-05	1.327	0.7075
RBC_157 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	17	Y = -2.04e-04x + 2.316	-2.04e-04	2.316	0.9933
RBC_157 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	18	Y = -3.14e-06x + 2.126	-3.14e-06	2.126	0.1580
RBC_178 - Air alvÃ©olaire	15/03/2021	Lun.	CoolTerm Capture 2021-03-15 08-31-36.txt	19	Y = 2.58e-05x + 1.334	2.58e-05	1.334	0.9423

Figure 45. *Results of EA measurements classified according to the date, weekday, the raw file, id, regression line, the slope as well as the ordinate and* R^2

OPEA database were constituted, then computational operations were performed by a dedicated statistician to average the triplicates values and convert the resulting raw average slope values into equivalent amount of H_2O_2 contained in the corresponding air sample, expressed as pmol/L_{air}.

Based on calibration curves (*refer part 3*), the linear relation used to convert the values was as follows:

$$[\mathrm{H2O2}]\mathrm{pmol/L\ air} = \frac{10\text{^}5.\,\mathrm{x\ slope}\ - 1.473}{0.0213}$$

Finally, for each participant, the OPEA final value was calculated as the ratio between the volunteer and ambient air values:

$$value\ in\ H202\ (pmol.L_{air}^{-1}) = [\mathrm{H2O2}](pmol.L_{air}^{-1})\mathrm{air\ ambiant} - \ [\mathrm{H2O2}](pmol.L_{air}^{-1})\ \mathrm{participant}$$

At the end of the medical check, the workers were provided with the air sampling pumps and direct-reading devices for monitoring airborne exposure during their working-shift. After the working-shift, participants were received again for the post-shift medical check including spirometry and biological sample collection. All spirometry tests were performed without bronchodilation by the same trained nurse. This standardized sequence of collecting samples, taking measurements and recording data were repeated for every worker daily for two weeks. It is worth mentioning that personal air samples were taken over the entire workday, including periods of service and breaks. A laboratory LEM technician assigned to each participant wore all personal sampling pumps and devices because the participants themselves were not allowed to wear any devices that can potentially interfere with their job. The technicians closely followed their corresponding worker during his/her work-shift and wore the complete set of equipment. Each technician was equipped with a set of 5-6 instruments for personal exposure characterization (Figure 46) in a backpack closed to the personal breathing zone of the technician. Personal breathing zone measure was not physically possible in the cramped space for station agents and locomotive operators. Each technician filled in the workers' activity logbook to document all its tasks, their duration, and the place of their realization during each work-shift. These logbooks thus provided contextual information for linkage with personal exposure measurement data.

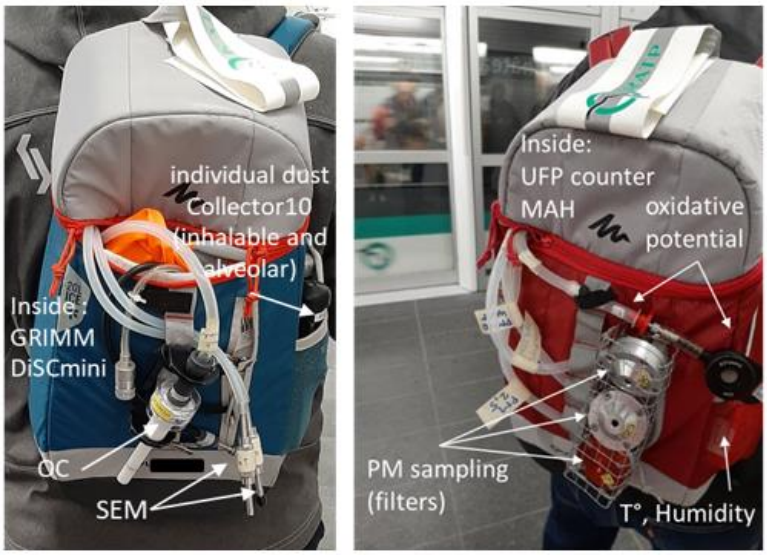


Figure 46. *Two RATP technician equipped with a) personal samplers for gravimetry (PM2,5; PM10; Cr III), oxidative potential (particle and volatile fractions) and monocyclic aromatic hydrocarbons (MAH) as well as direct reading instruments for relative humidity and temperature (EcoLog). b) Ultrafine particles (DiSCmini), OC (PM_4), GRIMM, individual dust collector Cip10 and SEM*

Several stationary equipment for VOC (Gasmet) and nanoparticles exposure characterization (DiSCmini, Nanomonitor) as well as hygrometry, temperature measurements (Ecolog) were also carried out at different target locations (Chatelet Station, Porte de la Villette station) (Figure 47).

Figure 47. *Direct-reading equipment at Châtelet place*

3. Description of exposure devices

A particle counter (“DISCmini”, (Testo, Mönchaltorf, Suisse) was used to measure particles from 10 to 300 nm, particle number concentration (#/cm3), and lung deposited specific area (LDSA) (recorded every 10 s). Both particle size and number concentrations are exposure metrics related to adverse health outcomes of ultrafine particles (UFP). LDSA corresponds to the probability of particles in the lung both for the alveolar and the tracheobronchial region following by ICRP model. The detection limit of DiSCmini was around 1,000,000 pt/cm3.

Fine particle size distribution between 250 nm to 32 µm and number concentration were measured with GRIMM. The GRIMM contained four different measurements parameter included particle number concentration, mass concentration, occupational health (inhalable, thoracic and respirable fraction) and environmental (PM_1, $PM_{2.5}$, and PM_{10}).

For $PM_{2.5}$, PM_{10} and their metal constituents, the sampling train consisted of filter (PTFE Membrane Filters (37 mm), Sigma-Aldrich, France) in a cassette holder (Personal Impactor H-PEM, BGI, USA connected to a cyclone and attached with flexible tubing to a pump (GilAir Plus, Sensidyne, Germany) operating at 4L/min.

Inhalable and respirable dusts were collected actively (pump at 10L/min) on foam using the individual dust samplers CIP 10-I and CIP 10-R, respectively.

Quantification of mass concentration (µg/m^3) was determined using standard gravimetric analysis for total inhalable and respirable dust and for $PM_{2.5}$ and PM_{10}. Particle mass concentration in $PM_{2.5}$ and PM_{10} were analyzed for 11 elements (Al, As, Ba, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) using two acidic digestion steps (95°C; HCl 30% for 25 min then HNO3 65% for 15 min) and inductively coupled plasma mass spectroscopy (ICP-MS). The measured concentrations were integrated over sampling time, which was equal to the 6-h work-shift duration. All laboratories were accredited for regulatory purpose analysis and used certified methods.

Airborne nanoparticles particles for microscopy analyses were collected onto SEM grid Q310AR-14 (Quantifoil R 1/4, 300 Mesh, Gold, Quantifoil Micro Tools GmbH, Germany) using Mini-Particle Sampler, MPS® (INERIS, France) with sampling flowrate 0.3 L/min for 10 min.

Random grid surfaces were analyzed with a transmission electronic microscopy (TEM) (CM100 Biotwin, at 80kV, Philips) and a scanning electronic microscopy (SEM) (Phenom ProX, at 15kV, Thermo Scientific) coupled with Energy-Dispersive X-ray detector (EDX). Particle morphology, size and chemical composition were determined.

Airborne nanoparticles (numbers/cm3) were measured with using a portable condensation particle Counter (Nanomonitor ITEN ®) with a sampling input that is cut off at 50% efficiency for an aerodynamic diameter of 2.5 µm.

Table 20. *Indoor air exposure measurements performed in the longitudinal pilot study in a Parisian subway*

Outcome measured	Aerosol fraction	Type of measurement	Sampling method (pump and filter/head)		Sampling duration	Analytical method
Inhalable and respirable dusts (environmental measures)	Inhalable and respirable	Personal	2 pumps: CIP10 I and CIP10 R	-	8h	Metropole m341
Fine particle mass concentration	Inhalable (PM_{10}, $PM_{2.5}$)	Personal	3 pumps at 4 L/min	Teflon filter	8h	Gravimetry (weighs before and after)
Metals (Fe, Cd, Al, Ba, Ni, Zn, Cu, Pb, Mn, As) mass concentration	Inhalable (PM_{10} and $PM_{2.5}$)	Personal	Same pumps as before	-	8h	The metals present in particles are collected on a filter with a diameter varying from 47 to 150 mm, then dissolved in an acidic medium using a microwave mineralizer (closed system). The liquid sample is then diluted and analyzed by ICP-MS
Metals Cr III and Cr VI	PM_{10}	Personal	Pumps at 4 L/min			
Organic carbon (OC)/elemental carbon(EC) mass concentration	Respiratory (PM_4)	Personal	1 pumps 2 L/min	Quartz filter - cassettes and cyclones + GALVIN (sealing filter for the cassette)	8h	The analytical technique includes thermo-optical processes for the separate determination of EC/OC contents, based on the successive and controlled combustion of the different particles deposited on the filters, according to a given temperature program, with optical correction of scale deposits.

Monocyclic Aromatic Hydrocarbons	Gaseos	Personal	1-2 pocket pumps 0,2 L/min	Activated charcoal	8h	(Benzene, Xylene (m, o, p), Toluene, 1,2,4 TMB)
Oxidative potential	Inhalable particles and gaseous	Personal	1 pump 2 L/min	Teflon filter (and storage cassette) + XAD2 adsorbent	8h	1) immersion of the filter in Fox 2) chemical desorption in dichloromethane - solvent evaporation - recovery in DMSO - injection in FOX
Ultrafine particle count, size and LDSA	Respirable ($PM_{0.1}$ (10nm-300nm))	Personal & stationary	5 DISCmini	Impaction head	6-8h	DISCmini, a portable personal direct-reading exposure monitor with a time resolution of 1s.
Particle morphology and elemental composition	Respirable (PM_1 and $PM_{0.1}$)	Personal	1 pump 0.2 L/min	1 mini-Sampler (holding head) + grid filter (SEM)	10min	Scanning electronic microscopy coupled with EDX (diffraction and impaction of x-rays on particles)
Fine and ultrafine particle mass concentration	PM_{10}, $PM_{2.5}$, PM_1 (300nm-32µm)	Personal	1 GRIMM	Impaction head	8h	Direct-reading optical particles counter Grimm 1.109, 31 channels, with a time resolution of 10s.
Volatile organic compounds*		Stationary			8h	Direct-reading device Gasmet
Hygrometry and temperature		Personal & stationary	2 Ecolog (ELPRO®)		8h	Direct-reading device Ecolog (ELPRO®)
Ultrafine particle count		Stationary	Nanomonitor (ITEN ®)		8h	Direct-reading device Nanomonitor

EC= elemental carbon, ICP-MS =Inductively Coupled Plasma Mass Spectrometry LDSA= Lung Deposited Surface Area OC=Organic carbon, PM= particulate matter, UFP= ultrafine particles. * Water vapor, Carbon dioxide, Carbon monoxide, Methane, Toluene, Formaldehyde, Acetone, Nitrogen dioxide, Nitrogen monoxide, Isopentane, Butane, Isobutane, Ethylène, o-xylène, P-xylène, m-xylène, Styrène, Acétaldéhyde, 1-pentanol, Dimethyl disulfide.

Table 21. *Biomarker measures performed in the longitudinal pilot study in the Parisian subway*

Outcome measured	Biological matrix	Analytical method
Biomarkers of exposure		
Transition metals (Fe, Cu, Zn, Mn, Co, Cr, Ni, Mo, Ti,V), Ba, Sb, S, Si, Al, Pb mass concentration	EBC	ICP-MS
Nanoparticles number concentration and mean hydrodynamic diameter	EBC	Nanoparticle Tracking Analyzer (Malvern)
Transition metals (Fe, Cu, Zn, Mn, Co, Cr, Ni, Mo, Ti,V), Ba, Sb, Si, Al, Pb mass concentration	Urine	ICP-MS
PAH metabolites (1-hydroxypyrene)	Urine	HPLC-Fluorescence
Biomarkers of effect		
OPEA	Alveolar and bronchial part of the exhaled air	OPEA analyzer + FOX colorimetric test (6 min including sampling)
MDA	EBC	LC-MS/MS
8-isoprostane and 8-OHdG	EBC	LC-MS/MS
$NO2^-$, $NO3^-$ and formate ion (nitrosant stress)	EBC	Ion-chromatography
Acetate, pyruvate, lactate, butyrate, propionate (cell metabolism)	EBC	Ion-chromatography
MDA	Urine	LC-MS/MS
8-isoprostane and 8-OHdG	Urine	LC-MSMS
Creatinine	Urine	LC-MS/MS

FOX= ferrous ion oxidation, ICP-MS= inductively coupled plasma mass spectrometry, LC-MS= Liquid chromatography–mass spectrometry, OPEA=oxidative potential exhaled air

In contrast to the pilot study, the **field study** focused only on the biomarkers pre-selected in the pilot study as well as health outcomes, which were measured only once per worker. The field study started on 15 March and ended on 7 May 2021, for a total of 8 campaign weeks. All workers were asked to fill in a standardized questionnaire, very similar to the questionnaire used in the pilot study. COPD diagnosis was the principal clinical outcome. COPD assessment was based on the 2017 update of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) Guidelines [262]. Here, COPD is defined by incompletely reversible airways obstruction—that is, a ratio of the post-bronchodilator forced expiratory volume in 1 second to the forced vital capacity (FEV_1/FVC ratio) of less than 70% in presence of the clinical symptoms such as dyspnea, chronic cough or sputum production. The GOLD guidelines recognize that the use of fixed FEV_1/FVC ratio values will result in more frequent COPD diagnosis in elderly, and in less frequent diagnosis among adults younger than 45 years, especially for mild COPD. The lower limit of normal (LLN) values of FEV_1/FVC, based on the normal distribution, classify the bottom 5% of healthy population as abnormal [262]. Considering the age-distribution within the cohort of the Parisian transport company workers [263], we used the LLN values of FEV_1/FVC as a second definition of COPD. For these LLN values, spirometric reference values determined from multi-ethnic reference values for spirometry for the 3-95-yr age range (the global lung function 2012 equations) will be used [264]. The same well-trained occupational physician performed spirometry to avoid inter-assessor differences. For every obstructive syndrome suspicion, a reversibility test by bronchodilation was performed, to make a differential diagnosis between COPD and asthma. This and secondary health outcomes of interest are described in Table 2. The course of the typical medical check for the field study is depicted in Figure 2. The volunteers came in groups of three to the occupational health center located at Porte de Bagnolet. The campaign of individual medical checks, biological sample and data collection had a maximum duration of the medical check of 2 hours per participant. We used the same methods for EBC, exhaled air and urine collection as those used in the pilot study, except that we have used spot urine samples and performed one more biological examination using two single use cytobrushes (Cepilo cervical cell sampler, Deltalab S.L.U., Spain, cat. No. 440150) for harvesting buccal cells (< 5 minutes) (Figure 48).

Figure 48. *Micronuclei frequency from cell in buccal mucosa sampling*

Table 22. *The course of the typical medical visit in the field study*

<table>
<tr><th></th><th>Volunteer 1</th><th>Volunteer 2</th><th>Volunteer 3</th></tr>
<tr><td>5min</td><td rowspan="6">Welcoming the participant and obtaining written consent - questionnaire</td><td rowspan="6">Welcoming the participant and obtaining written consent - questionnaire</td><td rowspan="6">Welcoming the participant and obtaining written consent - questionnaire</td></tr>
<tr><td>10min</td></tr>
<tr><td>15min</td></tr>
<tr><td>20min</td></tr>
<tr><td>25min</td></tr>
<tr><td>30min</td></tr>
<tr><td>35min</td><td rowspan="5">allergic skin test</td><td rowspan="3">First respiratory functionnal exploration</td><td>Total OPEA measure</td></tr>
<tr><td>40min</td><td>collection of cells from the buccal mucusa</td></tr>
<tr><td>45min</td><td>Alveolar OPEA measure</td></tr>
<tr><td>50min</td><td colspan="2">Total OPEA measure</td></tr>
<tr><td>55min</td><td>Alveolar OPEA measure</td><td>collection of cells from the buccal mucusa</td><td rowspan="2">First respiratory functionnal exploration</td></tr>
<tr><td>1h</td><td rowspan="4">Collection of exhaled breath condensate</td><td colspan="2">Alveolar OPEA measure</td></tr>
<tr><td>1h05</td><td rowspan="4">Collection of exhaled breath condensate</td><td rowspan="4">allergic skin test</td></tr>
<tr><td>1h10</td></tr>
<tr><td>1h15</td></tr>
<tr><td>1h20</td></tr>
<tr><td>1h25</td><td>Total OPEA measure</td><td rowspan="5">allergic skin test</td></tr>
<tr><td>1h30</td><td>collection of cells from the buccal mucusa</td><td rowspan="5">Collection of exhaled breath condensate</td></tr>
<tr><td>1h35</td><td rowspan="3">First respiratory functionnal exploration</td></tr>
<tr><td>1h40</td></tr>
<tr><td>1h45</td></tr>
<tr><td>1h50</td><td>Urine</td><td>Urine</td><td>Urine</td></tr>
<tr><td>1h55</td><td colspan="3" rowspan="5">inhalation of a bronchodilator + 2nd respiratory functional exploration</td></tr>
<tr><td>2h</td></tr>
<tr><td>2h05</td></tr>
<tr><td>2h10</td></tr>
<tr><td>2h15</td></tr>
</table>

Different steps are classified in order of completion and in duration time - The steps could be switched or condensed (e.g Exhaled breath and skin tests at the same time) depending on the situation – three volunteers arrived at the same time. (For a total of 12 volunteers per day) and were assigned to three different rooms for the duration of the medical examination.

4. Statistical analysis

Data collected within the pilot study were analyzed using an exploratory approach. Descriptive analysis gives information on the cumulative trends of exposures to different PM fractions, their metal content and other airborne chemicals. Direct-reading fine and ultrafine particle exposure measurement data (from DISCmini and Grimm, Table 20) were analyzed along with data from the activity logbooks, using time series analytical technics such as Bayesian spline analysis to get insights on the exposure sources and determinants.

Repeated data were analyzed using mixed linear models and interval regression models for the censured data (below LOD/LOQ). Air and biomarker concentrations were compared between occupations, using daily, pre- and post-shift log-transformed mean values with and without adjustment for variables collected through the standardized questionnaire (e.g., age, average home-to-work commute time, anti-oxidant diet, non-occupational exposure to PM). Correlation between external and internal exposure (based on urine and EBC biomarkers of exposure), internal exposure and early effect biomarkers, and the latter with FEV_1/FVC were tested, using post-shift to pre-shift values ratios for each biomarker. Analysis was conducted applying different lag-times on the exposure estimates to explore the temporal variation in biomarker levels. The most informative biomarkers (i.e., correlated both with exposure and particularly with FEV_1/FVC ratio) were selected for the field study.

In the field study, the central research hypothesis is that a panel of oxidative stress biomarkers measured in non-invasive samples, including OPEA will be more efficient for COPD diagnosis than single biomarker analysis. This hypothesis was tested using generalized linear models. Besides, additional associations between dependent variables and explanatory variables were examined, as shown in Figure 49. Data management and statistical analyses were performed a dedicated statistician using Stata, version 16, software.

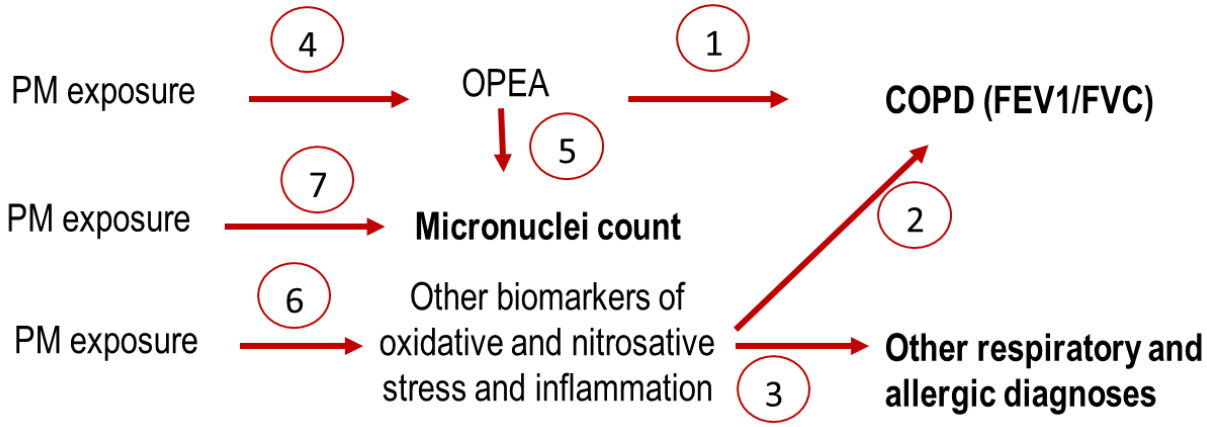


Figure 49. *Associations to be considered during statistical analysis of the field study data, numbered by order of investigation. The dependent variables corresponding to the studied biological or health effects are shown in bold*

Table 23. *Primary and secondary health outcome definition and measurement in the field study in the Parisian subway*

Outcome	Measurement method	Diagnostic criteria
Chronic Obstructive Lung Disease	Spirometry performed by the trained occupational physician with the reversibility test if necessary	Post-bronchodilator forced expiratory volume in 1 s to the forced vital capacity (FEV_1/FVC ratio) of less than 70% in presence of the clinical symptoms such as dyspnea, chronic cough or sputum production
Micronuclei frequency	Micronuclei counting (per 1000 cells (‰)) according to the standardized protocol (Buccal Cell Micronuclei Assay)	The individual measured values were interpreted using a frequency diagram of micronuclei versus age in the healthy unexposed population [70]. Values above reference values for a given age were considered an early signal of the effect of genotoxic exposure
Asthma	Self-administered questionnaire	Being on treatment for asthma or at least one of the symptoms suggestive of asthma (asthma attack(s), wheezing in the chest, difficulty in breathing, attack of breathlessness) in the past 12 months
Chronic bronchitis	Self-administered questionnaire	Productive cough for at least 3 consecutive months per year and for at least 2 consecutive years or when the diagnosis has been confirmed by a physician
Emphysema	Self-administered questionnaire	Diagnosis has been confirmed by a physician
Active allergic rhinitis	Self-administered questionnaire	Symptomatic allergic rhinitis (sneezing or a runny or stuffy nose without having a cold or the flu) and under treatment during the last 12 months
Eczema	Self-administered questionnaire	Eczema diagnosis and/or treatment in the past 12 months
Atopy	Immediate reading allergic skin tests by the prick-test technique for 12 most common pneumallergens	The test results were read by the occupational physician and considered positive if the diameter of the papule formed is greater than or equal to 3 mm and at least equal to half of the papule of the positive control (histamine)

III. Pilot study results

1. My contribution to the pilot study

At this point, I was thoroughly involved with the organization/management of all environment sampling and with the train of RATP staff to perform specific biological sampling (urine and EBC), in the observance of best practices guidelines when collecting and storing biological samples in the occupational setting. Finally, I was in charge of analyzing the MDA concentration in EBC and computing the OPEA measurements.

2. Valorisation of the pilot study results

The different results of this study have been valorized in the form of four articles:

- Analysis of the determinants of occupational exposure to ultra fine particles (related to DiSCmini)

T. Ben Rayana, **M. Hemmendinger,** C. Crézé, P. Wild, J.-J. Sauvain, G. Suarez, S. Besançon, N. Méthy, K. Sakthithasan, G. Carillo, A. Debatisse, V. Jouannique, B.C. Guinhouya, I. Guseva Canu, Analyse exploratoire des mesures de particules ultrafines en temps réel dans des enceintes ferroviaires souterraines de transport public, Archives des Maladies Professionnelles et de l'Environnement, 2022, 10.1016/j.admp.2022.01.005

- Assessment of the real-time UFP number concentration (related to DiSCmini) in the personal breathing zone of the nine subway professionals

Petremand, Remy & Wild, Pascal & Crézé, Camille & Suárez, Guillaume & Besançon, Sophie & Jouannique, Valérie & Debatisse, Amélie & Guseva Canu, Irina. (2021). Application of the Bayesian spline method to analyze real-time measurements of ultrafine particle concentration in the Parisian subway. Environment International. 156. 106773. 10.1016/j.envint.2021.106773.

- Quantification of particulate matter concentrations and metal concentrations in EBC, urine, and PM and investigation of their associations.

I. Guseva Canu, C. Crézé, **M. Hemmendinger,** T. Ben Rayana, S. Besançon, V. Jouannique, A. Debatisse, P. Wild, J.J. Sauvain, G. Suárez, N.B. Hopf, Particle and metal exposure in Parisian subway: Relationship between exposure biomarkers in air, exhaled

breath condensate, and urine, International Journal of Hygiene and Environmental Health, Volume 237, 2021, 113837, 10.1016/j.ijheh.2021.113837.

- Association between PM exposure and nitrosative/ oxidative stress markers and/or metabolic biomarkers (acetate, lactate, formate) measured in the EBC.

Jean-Jacques Sauvain, **Maud Hemmendinger**, Guillaume Suárez, Camille Creze , Nancy B. Hopf, Valérie Jouannique, Amélie Debatisse, Jacques A. Pralong, Pascal Wild and Irina Guseva Canu, Malondialdehyde and anion patterns in exhaled breath condensate among subway workers, Particle and Fibre Toxicology 2022 19:16, 10.1186/s12989-022-00456-z

3. Summary of the main results from pilot study

a. Description of study sample

A total of nine workers were enrolled in this pilot study. Three different professional groups were included: station agents, locomotive operators and security guards. All three station agents were women. Participants were 47-year-old on average (± standard deviation (SD) 9.42), the youngest was 30.5-year-old and the oldest 58-year-old. They had worked for the company for 14.66 ± 7.19 years on average, the shortest employment duration was 3 years and the longest 25 years. The majority (55.56%) of participants reported home-to-work commuting time less than an hour. For those spending more than an hour on their home-to-work commuting, spent 86.25 ± 19.31 minutes on average, the longest duration recorded was 1 hour and 45 minutes. Nobody reported using a bicycle for their home-to-work commute, 22% used a motorbike or a car, and the other 78% used public transportation (subway (67%) or local train (33%)). Only 33% of participants reported walking when commuting from home to work or vice-versa. Commuting habits were similar regardless the commuting time (more or less than an hour). The average sitting duration was 5.27 hours/day with minimum duration of 1 hour and 15 minutes and the maximum of 12 hours. Regarding the life-style and eating habits, most (78%) participants reported no particular diet or vitamin or food supplements. All reported drinking alcohol, particularly vine (77%), but also other alcoholic beverage (33%); 33% of participants reported every-day alcohol consumption. At home, all participants used interior fragrance candles, and 55.56% used them every day. All participants self-declared as healthy. On the 8-point Likert scale of general health (1=excellent and 8=very bad), all participants ranked their health below or equal to 4, the majority (66.67%) even graded it 1 or 2. The average body mass index (BMI) of participants was 25.54 ± 2.28, but differed by

sex. Female participants were all of healthy weight (mean BMI= 23.70 ± 1.95), while men were overweight (mean BMI= 26.47 ± 1.94), although none was obese. Three participants reported having some respiratory/allergy problems, and two to be hypertensive. All participants completed the exposure follow-up and provided 18 or 20 EBC and urine samples. The biological samples were of adequate quality resulting in 168 EBC and 168 urines samples in total. Creatinine concentration was within normal range in 144 urine samples (85.71%). In 17 samples (10%) creatinine concentrations were below 0.5 g/L, mostly (82.35%) in the post-shift urine samples, while in 7 samples (4%) above 3 g/L and for 57.14% of the samples in the pre-shift urine.

b. Exposure levels of the three professional categories.

Their personal daily exposure to PM_{10}, PM_{4}, $PM_{2.5}$ and ultrafine fraction as well as their metal content was measured for two consecutive working weeks. Exposure concentrations are described in detail in Table 24 and compared either to regulatory values when they exist, or to guide values in the literature. PM concentrations irrespective of particle size were lowest among station agents and highest among locomotive operators. Station agents were exposed to the highest number concentrations of UFP, but converted to LDSA metrics, locomotive operators had the highest exposure (44.79 µm2/cm3, CI95% = 39.36–50.21) closely followed by station agents (37.79 µm2/cm3, CI95% = 28.99–46.59).

The locomotive operators had the highest exposure to PM_{10} and $PM_{2.5}$ and their concentrations were slightly higher than French regulatory values. The PNOS concentrations were below current French regulatory values; 10 mg/m3 and 5 mg/m3 for inhalable and respirable particles, respectively.

Fifty percent of the values for six (As, Ba, Cd, Cr, Ni, Pb) of the 11 metals quantified in PM_{10} were below LOQ.

Table 24. *Estimated geometric concentration (with 95% confidence interval values into bracket) of the different fractions (ultrafine, $PM_{2.5}$, respirable and inhalable dusts) measured for the three different professional groups, as well as the UFP: Ultrafine particle number, size and lung deposited surface area. Concentration of different elements (aluminium, iron, copper, manganese, arsenic, cadmium, chrome, lead, nickel, barium and zinc) in the PM_{10} and $PM_{2.5}$ fraction. Concentration of organic and elemental carbon in respirable fraction*

Parameter measured	Predicted mean concentration (95% confidence interval) accorded to the model	Predicted mean concentration (95% confidence interval) accorded to the model	Predicted mean concentration (95% confidence interval) accorded to the model	French regulatory values	Guide values
	Station agents (V1-V2-V3)	Locomotive operators (V4-V5-V6)	Security guards (V7-V8-V9)		
Respirable dusts (mg/m3)	0.07 (0.06 - 0.08)	0.10 (0.07 - 0.12)	0.09 (0.07 - 0.11)	if PNOS (e,g, Al, Mg) - 5 (mg/m3) 8-hour average exposure	-
Inhalable dusts (mg/m3)	0.06 (0.05 - 0.08)	0.20 (0.15 - 0.26)	0.14 (0.10 - 0.18)	if PNOS (e,g, Al, Mg) - 10 (mg/m3) 8-hour average exposure	-
PM2.5 (µg/m3)	44.49 (25.47 - 63.52)	136.83 (70.04 - 203.62)	47.79 (22.22 - 73.37)	10 (µg/m3) on average per year or 25 (µg/m3) as a daily average	5 (µg/m3) on average per year or 15 (µg/m3) as a daily average (WHO)
PM10 (µg/m3)	54.20 (36.37 - 72.03)	188.50 (101.93 - 275.07)	79.71 (43.95 - 115.46)	20 (µg/m3) on average per year or 50 (µg/m3) as a daily average	15 (µg/m3) on average per year or 45 (µg/m3) as a daily average (WHO guide values 2022) // 311 (µg/m3) for the 2h15 exposure of subway users in underground railway (French higher council of public hygiene guide values)
DiSCmini				-	-
UFP Number (#/cm3)	20’000 (16’000-24’000)	15’000 (12’000-17’000)	8’900 (7’000-11’000)	-	-
UFP Size (nm)	37 (35–39)	50 (47–52)	49 (46–52)	-	-
UFP LDSA (µm2/cm3)	37,79 (28,99–46,59)	44,79 (39,36–50,21)	25,45 (22,19–28,71)	-	-

Al in PM2.5 (µg/m3)	5.76	(3.77	-	7.76)	4.24	(2.08	-	6.41)	7.74	(3.73	-	11.75)	-	VLEP-8h global metal or trioxide Al 10 000 (µg/m3)
Fe in PM2.5 (µg/m3)	1.48	(0.98	-	1.97)	15.65	(9.03	-	22.26)	5.11	(2.60	-	7.63)	-	VLEP-8h global trioxide Fe 5000 (µg/m3)
Zn in PM2.5 (µg/m3)	0.40	(0.34	-	0.46)	0.64	(0.50	-	0.78)	0.40	(0.31	-	0.49)	-	VLEP-8h global Zn oxide 5000 (µg/m3)
Ba in PM2,5 (µg/m3)		< LOQ				< LOQ				< LOQ			OEL-8h global Ba-based compounds 500 (µg/m3)	-
As in PM2,5 (µg/m3)		< LOQ				< LOQ				< LOQ				OEL-8h global trioxide As 200 (µg/m3)
Cd in PM2,5 (µg/m3)		< LOQ				< LOQ				< LOQ			OEL-8h respirable and inhalable Cd 4 (µg/m3)	-
Cr in PM2,5 (µg/m3)		< LOQ				< LOQ				< LOQ			global metal, inorganic and Cr-based compounds 2000 (µg/m3)	-
Cu in PM2,5 (µg/m3)		< LOQ				< LOQ				< LOQ			-	OEL-8h global Cu dusts 1000 (µg/m3)
Mn in PM2,5 (µg/m3)		< LOQ				0,22 (0,20-0,24)*				0,12 (0,07-0,17)*			OEL-8h respirable Mg 50 (µg/m3), inhalable Mg 200 (µg/m3)	-
Ni in PM2,5 (µg/m3)		< LOQ				< LOQ				< LOQ			OEL-8h global Ni oxides 1000 (µg/m3), Ni sulfate 0,1 (µg/m3)	Inhalable Ni : 100 (µg/m3) (ECHA)
Pb in PM2,5 (µg/m3)		< LOQ				< LOQ				< LOQ			OEL-8h global metallic Pb 100 (µg/m3)	-
													-	-
Al in PM10 (µg/m3)	5.20	(3.38	-	7.02)	6.61	(2.97	-	10.26)	13.22	(6.27	-	20.17)	-	OEL-8h global metal or trioxide Al 10 000 (µg/m3)
Fe in PM10 (µg/m3)	2.49	(1.23	-	3.75)	48.11	(21.72	-	74.50)	12.32	(3.40	-	21.25)	-	OEL-8h global trioxide Fe 5000 (µg/m3)
Zn in PM10 (µg/m3)	0.61	(0.49	-	0.73)	1.08	(0.76	-	1.39)	0.42	(0.30	-	0.54)		OEL-8h global Zn oxide 5000 (µg/m3)
Cu in PM10 (µg/m3)	0.05	(0.03	-	0.07)	0.15	(0.08	-	0.21)	0.15	(0.08	-	0.21)	-	OEL-8h global Cu dusts 1000 (µg/m3)

Mn in PM10 (µg/m3)	0.06	(0.05	-	0.08)	0.45	(0.32	-	0.59)	0.13	(0.09	-	0.17)	OEL-8h respirable Mg 50 (µg/m3), inhalable Mg 200 (µg/m3)	-
As in PM10 (µg/m3)	< LOQ				< LOQ				< LOQ				-	OEL-8h global trioxide As 200 (µg/m3)
Ba in PM10 (µg/m3)	< LOQ				0,38 (0,33-0,43)*				0,22 (0,17-0,27)*				OEL-8h global Ba-based compounds 500 (µg/m3)	-
Cr in PM10 (µg/m3)	< LOQ				0,17 (0,14-0,20)*				< LOQ				OEL-8h global metal, inorganic and Cr- based compounds 2000 (µg/m3)	-
Cr III in PM10 (µg/m3)	< LOQ				0,17 (0,14-0,20)				< LOQ				OEL-8h Cr III- based compounds 2000 (µg/m3)	-
Cr VI in PM10 (µg/m3)	< LOQ				< LOQ				< LOQ				OEL-8h global Cr VI-based compounds 1 (µg/m3)	-
Cd in PM10 (µg/m3)	< LOQ				< LOQ				< LOQ				OEL-8h respirable and inhalable Cd 4 (µg/m3)	-
Ni in PM10 (µg/m3)	< LOQ				< LOQ				< LOQ				OEL-8h global Ni oxides 1000 (µg/m2), Ni sulfate 0,1 (µg/m3)	respirable : 5 (µg/m3), inhalable : 30 (µg/m3) (ECHA)
Pb in PM10 (µg/m3)	< LOQ				< LOQ				< LOQ				OEL-8h global metallic Pb 100 (µg/m3)	-
OC respirable fraction (mg/m3)	0,05 (0,04-0,06)*				0,08 (0,06-0,11)*				0,04 (0,04 -0,05)*				-	-
EC respirable fraction (mg/m3)	< LOQ				< LOQ				< LOQ				from 21/02/2023 - OEL-8h 0,05 (mg/m3) 8-hour average exposure	-
HAP	NA				NA				NA				-	-

*There are no regulations for metals in $PM_{2.5}$ and PM_{10}, so the values presented here are from other fractions. *non-predicted by the model [265] . PNOS = particles not otherwise specified, are defined as dusts that do not show any effect other than those resulting from the consequences of pulmonary overload, unless a specific effect can be demonstrated, V= volunteer, OEL-8h: 8-hour average professional exposure, OC = organic carbon, EC= elemental carbon. Missing limits are indicated by a dash.*

PM_{10} contained up to 40% Fe and 20% Al, and less than 2% Cu, Zn and Mn and varied significantly across jobs (Table 24). The locomotive operators had the highest exposure to Fe (irrespective of particle size), while the security guards had the highest exposures to Al.

Table 25. *Proportion of different elements (aluminum, iron, copper, manganese and zinc) in the PM_{10} and $PM_{2.5}$ fraction*

	Predicted marginal mean (95% Confidence Interval)					
	Station agents		Locomotive operators		Security guards	
Ratio Al $PM_{2.5}$	0,17	(0,06- 0,29)	0,04	(0,01-0,06)	0,18	(0,04-0,31)
Ratio Al PM_{10}	0,19	(0,02-0,36)	0,06	(-0,01-0,12)	0,22	(-0,03-0,46)
Ratio Fe $PM_{2.5}$	0,04	(0,02-0,06)	0,12	(0,05-0,20)	0,12	(0,04-0,20)
Ratio Fe PM_{10}	0,08	(0,02-0,15)	0,39	(0,04-0,74)	0,21	(0,01-0,40)
Ratio Zn $PM_{2.5}$	0,01	(0,00-0,02)	0,00	(0,00-0,01)	0,01	(0,00-0,02)
Ratio Zn PM_{10}	0,01	(0,01-0,02)	0,01	(0,00-0,01)	0,01	(0,00-0,01)
Ratio Cu PM_{10}	0,00	(0,00-0,00)	0,00	(0,00-0,00)	0,00	(0,00-0,01)
Ratio Mn PM_{10}	0,00	(0,00-0,00)	0,00	(0,00-0,01)	0,00	(0,00-0,00)

c. Averaged EBC level of the considered variables

Particle number concentrations in EBCs were dependent on sampling time point (higher in pre-shift EBC than in post-shift). The concentration in descending order of the quantified elements in EBC was Zn > Cu > Ni > Mn. Iron was a predominant element in the collected underground particles (up to 40% for PM_{10}, Table 25), but it could not be consistently quantified in the EBC samples. Only 25% of the samples presented iron levels above the limit of quantification (LOQ) of 1 µg/L and 59% had concentration comprised between LOQ and limit of detection (LOD), set at 0.3 µg/L.

The averaged concentration of sub-micrometer particles in EBC (using nanoparticle tracking analysis - NTA was relatively low, comprised between 29-46.10^6 particles/mL, with a mean size distribution between 135–150 nm. Locomotive operators had the highest concentration of sub-micrometer particles in EBC compared to other subway professionals.

While 8-isoprostane and 8-OHdG could not be detected in the samples, MDA could be measured in all professional groups, and most concentrations fell within the concentration range reported in healthy controls. Station agents exhibited the highest MDA concentrations (160 ng/L, CI95%= 79-240 µg/L) (Figure 50) and decreased for locomotive operators and security guards, but without reaching a statistical significance (p=0.062).

Table 26. *Estimated geometric concentration or average concentration ± 95% confidence interval into bracket of the selected variables in EBC samples collected during the two working weeks for the three professional categories. The concentrations are compared either to regulatory values when they exist, or to guide values in the literature*

Parameter measured		Mean concentration (95% confidence interval)*				Predicted mean concentration (95% confidence interval) accorded to the model
		V1	V2	V3	**Shift**	Station agents
NTA	**LOD**					
Particles number concentration (10^6 #/mL)	26	72,15 (35,37-108,92)	42,18 (17,74-66,61)	49,61 (33,12-66,16)	pre-shift post-shift	31,34 (20,02- 42,66) 22,77 (14,4- 31,13)
Median particle diameter (nm)		127,53 (115,94-139,12)	149,01 (132,68-165,34)	140,62 (126,03-155,21)	post-shift	138,3 (122- 154,5)
Metal concentration						
Zn (ng/ml)	0,33	8,69 (6,53-10,86)	9,90 (5,64-14,15)	20,86 (7,17-34,56)	pre-shift post-shift	8,65 (6,00- 11,30) 10,25 (7,47- 13,03)
Cu (ng/ml)	0,07	0,86 (0,67-1,05)	0,86 (0,44-1,28)	1,44 (0,80-2,08)	pre-shift post-shift	0,98 (0,71- 1,25) 0,79 (0,60- 0,99)
Ni (ng/ml)	0,01	0,33 (0,23-0,43)	0,21 (0,09-0,33)	0,78 (0,30-1,26)	pre-shift post-shift	0,41 (0,26- 0,57) 0,23 (0,15- 0,30)
Ba (ng/ml)	0,01	0,26 (0,16-0,37)	0,25 (0,12-0,38)	0,32 (0,08-0,57)	pre-shift post-shift	0,21 (0,13- 0,29) 0,21 (0,14- 0,28)
Mn (ng/ml)	0,01	0,13 (0,10-0,16)	0,19 (0,11-0,27)	0,14 (0,11-0,18)	pre-shift post-shift	0,12 (0,08- 0,17) 0,14 (0,09- 0,19)
Cr (ng/ml)	0,01	0,03 (0,01-0,04)	0,07 (0,00-0,14)	0,04 (0,02-0,06)	pre-shift post-shift	0,02 (0,01- 0,03) 0,03 (0,02- 0,04)
Co (ng/ml)	0,01	0,04 (0,03-0,05)	0,04 (0,02-0,07)	0,04 (0,03-0,05)	pre-shift post-shift	0,04 (0,03- 0,04) 0,04 (0,03- 0,04)
Sb (ng/ml)	0,01	0,02 (0,02-0,03)	0,02 (0,01-0,03)	0,03 (0,02-0,04)	pre-shift post-shift	0,02 (0,01- 0,03) 0,02 (0,01- 0,03)
Pb (ng/ml)	0,01	0,06 (0,04-0,09)	0,04 (0,02-0,06)	0,10 (0,04-0,15)	pre-shift post-shift	0,06 (0,04- 0,08) 0,04 (0,03- 0,06)
Al (ng/ml)	1,63	3,03 (2,59-3,47)	3,15 (2,39-3,90)	3,28 (2,77-3,78)	-	<LOQ
Fe (ng/ml)	0,3	0,87 (0,56-1,18)	1,06 (0,62-1,51)	0,87 (0,53-1,22)	-	<LOQ
Mo (ng/ml)	0,01	<LOD	<LOD	<LOD	-	<LOD
Si (ng/ml)	7	16,70 (12,77-20,62)	14,11 (10,48-17,75)	17,59 (9,58-25,61)	-	<LOQ
Ti (ng/ml)	0,33	<LOD	<LOD	<LOD	-	<LOD
V (ng/ml)	0,01	<LOD	<LOD	<LOD	-	<LOD
Biomarkers						
MDA (pg/ml)	72	92,20 (71,50-113,24)	240,21 (168,26-312,16)	403,39 (293,07- 513,70)	-	160 (79-240)
8-isoprostane (pg/ml)	1	<LOD	<LOD	<LOD	-	<LOD
8-OHdg (pg/ml)	0,5	<LOD	<LOD	<LOD	-	<LOD
					-	
Acetate (µM)	0,27	60,02 (47,51-72,53)	40,28 (33,84-46,71)	45,27 (37,29-53,24)	-	44,6 (20,1-69,1)
Propionate (µM)	0,07	10,18 (7,91-12,44)	8,62 (7,16-10,08)	12,09 (9,20-14,98)	-	9,28 (5,65-12,92)
Butyrate (µM)	0,58	0,96 (0,84-1,08)	1,52 (1,27-1,76)	1,13 (0,95-1,32)	-	1,12 (0,84-1,41)
Formate (µM)	0,12	1,48 (1,35-1,61)	2,08 (1,79-2,37)	2,73 (2,26-3,19)	-	1,95 (1,61-2,29)
Lactate (µM)	0,11	2,34 (0,88-3,81)	8,03 (3,14-12,91)	7,97 (2,68-13,26)	-	2,04 (0,92-3,16)
Pyruvate (µM)	0,16	<LOD	<LOD	<LOD	-	0,005 (-0,01-0,02)
Nitrite NO2-(µM)	0,2	1,50 (1,28-1,71)	2,15 (1,86-2,44)	1,85 (1,48-2,22)	-	1,71 (1,35-2,06)
Nitrate NO3- (µM)	0,07	0,66 (0,56-0,75)	1,18 (0,90-1,46)	1,22 (0,92-1,52)	-	0,90 (0,72-1,08)
sum NOx (µM)	-	2.71 (2.25-3.16)				
NO_2^-/NO_3^-	-	1.90 (1.46-2.34)				

Parameter measured		Mean concentration (95% confidence interval)*				Predicted mean concentration (95% confidence interval) accorded to the model		
		V4	V5	V6	**Shift**	Locomotive operators		
NTA	**LOD**							
Particles number concentration (10^6 #/mL)	26	63,76 (42,65-84,87)	51,35 (41,55-61,14)	47,71 (36,16-59,25)	pre-shift	45,78	(29,5-	62,02)
					post-shift	33,25	(21,3-	45,23)
Median particle diameter (nm)		143,47 (132,40-154,54)	148,26 (131,55-164,98)	149,80 (131,71-167,89)	post-shift	146,4	(130-	162,8)
Metal concentration								
Zn (ng/ml)	0,33	8,74 (5,53-11,96)	7,24 (6,14-8,34)	7,35 (6,69-8,01)	pre-shift	6,69	(4,95-	8,44)
					post-shift	7,58	(5,60-	9,56)
Cu (ng/ml)	0,07	0,97 (0,65-1,29)	0,94 (0,73-1,14)	1,02 (0,74-1,31)	pre-shift	0,75	(0,57-	0,93)
					post-shift	1,01	(0,77-	1,25)
Ni (ng/ml)	0,01	0,30 (0,21-0,38)	0,38 (0,14-0,62)	0,25 (0,15-0,34)	pre-shift	0,2	(0,14-	0,27)
					post-shift	0,3	(0,20-	0,39)
Ba (ng/ml)	0,01	0,18 (0,13-0,24)	0,27 (0,19-0,35)	0,28 (0,13-0,43)	pre-shift	0,23	(0,15-	0,30)
					post-shift	0,2	(0,13-	0,26)
Mn (ng/ml)	0,01	0,13 (0,09-0,17)	0,15 (0,12-0,18)	0,15 (0,12-0,18)	pre-shift	0,12	(0,08-	0,17)
					post-shift	0,14	(0,10-	0,19)
Cr (ng/ml)	0,01	0,06 (0,03-0,08)	0,04 (0,03-0,05)	0,04 (0,02-0,06)	pre-shift	0,04	(0,02-	0,05)
					post-shift	0,04	(0,03-	0,05)
Co (ng/ml)	0,01	0,04 (0,03-0,05)	0,04 (0,03-0,05)	0,04 (0,03-0,05)	pre-shift	0,04	(0,03-	0,04)
					post-shift	0,04	(0,03-	0,04)
Sb (ng/ml)	0,01	0,03 (0,02-0,03)	0,03 (0,02-0,03)	0,02 (0,01-0,03)	pre-shift	0,02	(0,01-	0,02)
					post-shift	0,02	(0,02-	0,03)
Pb (ng/ml)	0,01	0,07 (0,02-0,12)	0,04 (0,03-0,05)	0,08 (0,04-0,11)	pre-shift	0,03	(0,02-	0,04)
					post-shift	0,06	(0,04-	0,07)
Al (ng/ml)	1,63	3,05 (2,46-3,64)	2,09 (1,91-2,27)	2,57 (1,64-3,50)	-	<LOQ		
Fe (ng/ml)	0,3	0,90 (0,57-1,23)	0,93 (0,46-1,39)	1,54 (0,13-2,96)	-	<LOQ		
Mo (ng/ml)	0,01	0,02 (0,01-0,03)	<LOD	<LOD	-	<LOD		
Si (ng/ml)	7	10,66 (9,00-12,31)	13,40 (5,82-20,97)	12,91 (10,49-15,33)	-	<LOQ		
Ti (ng/ml)	0,33	<LOD	<LOD	<LOD	-	<LOD		
V (ng/ml)	0,01	<LOD	<LOD	<LOD	-	<LOD		
Biomarkers								
MDA (pg/ml)	72	83,90 (68,95-98,85)	106,78 (89,01-124,54)	144,48 (115,80-173,14)	-	100 (50-160)		
8-isoprostane (pg/ml)	1	<LOD	<LOD	<LOD	-	<LOD		
8-OHdg (pg/ml)	0,5	<LOD	<LOD	<LOD	-	<LOD		
					-			
Acetate (µM)	0,27	13,27 (8,27-18,27)	56,25 (47,17-65,32)	36,06 (25,86-46,25)	-	25,2 (11,3-39,1)		
Propionate (µM)	0,07	3,32 (2,14-4,50)	10,46 (8,62-12,29)	9,33 (5,12-13,54)	-	5,83 (3,530-8,13)		
Butyrate (µM)	0,58	0,58 (0,49-0,68)	1,15 (0,98-1,32)	1,02 (0,76-1,29)	-	0,78 (0,58-0,98)		
Formate (µM)	0,12	1,55 (1,41-1,69)	1,77 (1,63-1,91)	2,01 (1,79-2,23)	-	1,73 (1,43-2,04)		
Lactate (µM)	0,11	4,43 (1,55-7,30)	3,56 (2,02-5,11)	3,70 (1,79-5,61)	-	2,10 (0,93-3,28)		
Pyruvate (µM)	0,16	<LOD	<LOD	<LOD	-	0,001 (-0,02-0,003)		
Nitrite NO2-(µM)	0,2	0,89 (0,78-0,99)	1,62 (1,28-1,97)	1,54 (1,25-1,83)	-	1,23 (0,97-1,49)		
Nitrate NO3- (µM)	0,07	1,05 (0,67-1,43)	0,94 (0,73-1,15)	1,24 (0,84-1,63)	-	0,93 (0,74-1,12)		
sum NOx (µM)	-	2.28 (1.88-2.67)						
NO_2^-/NO_3^-	-	1.32 (1.00-1.64)						

Parameter measured		Mean concentration (95% confidence interval)*				Predicted mean concentration (95% confidence interval) accorded to the model		
		V7	V8	V9	**Shift**	Security guards		
NTA	**LOD**							
Particles number concentration (10^6 #/mL)	26	58,40 (42,89-73,91)	56,99 (43,19-70,79)	105,41 (63,75-147,07)	pre-shift post-shift	39,85 28,95	(25,8- (18,5-	53,9) 39,38)
Median particle diameter (nm)		134,23 (122,50-145,96)	133,80 (124,46-143,14)	183,73 (149,58-217,88)	post-shift	150,3	(134-	166,6)
Metal concentration								
Zn (ng/ml)	0,33	8,31 (7,15-9,48)	11,18 (8,33-14,03)	12,26 (8,52-15,99)	pre-shift post-shift	11,68 8,42	(8,65- (6,08-	14,72) 10,75)
Cu (ng/ml)	0,07	1,08 (0,79-1,37)	1,57 (0,96-2,19)	2,02 (1,02-3,03)	pre-shift post-shift	1,38 0,96	(1,06- (0,71-	1,70) 1,20)
Ni (ng/ml)	0,01	0,26 (0,15-0,36)	0,42 (0,19-0,66)	0,48 (0,30-0,67)	pre-shift post-shift	0,31 0,22	(0,21- (0,15-	0,41) 0,30)
Ba (ng/ml)	0,01	0,41 (0,23-0,59)	0,66 (0,10-1,21)	0,51 (0,25-0,77)	pre-shift post-shift	0,37 0,33	(0,25- (0,21-	0,48) 0,45)
Mn (ng/ml)	0,01	0,25 (0,03-0,47)	0,21 (0,12-0,30)	0,57 (0,24-0,91)	pre-shift post-shift	0,22 0,18	(0,15- (0,12-	0,30) 0,24)
Cr (ng/ml)	0,01	0,03 (0,03-0,04)	0,06 (0,02-0,09)	0,09 (0,03-0,16)	pre-shift post-shift	0,04 0,04	(0,03- (0,03-	0,06) 0,06)
Co (ng/ml)	0,01	0,04 (0,03-0,05)	0,03 (0,02-0,04)	0,04 (0,03-0,05)	pre-shift post-shift	0,03 0,04	(0,03- (0,03-	0,04) 0,04)
Sb (ng/ml)	0,01	0,02 (0,02-0,03)	0,03 (0,02-0,03)	0,04 (0,02-0,05)	pre-shift post-shift	0,03 0,02	(0,02- (0,02-	0,03) 0,03)
Pb (ng/ml)	0,01	0,05 (0,01-0,08)	0,06 (0,03-0,08)	0,06 (0,04-0,08)	pre-shift post-shift	0,05 0,03	(0,03- (0,02-	0,06) 0,04)
Al (ng/ml)	1,63	2,28 (2,00-2,56)	2,45 (1,89-3,01)	4,69 (3,04-6,35)	-	<LOQ		
Fe (ng/ml)	0,3	0,69 (0,42-0,96)	0,88 (0,51-1,25)	1,51 (0,50-2,52)	-	<LOQ		
Mo (ng/ml)	0,01	<LOD	0,13 (0,01-0,25)	0,12 (0,01-0,24)	-	<LOQ		
Si (ng/ml)	7	35,47 (22,75-48,20)	12,80 (9,99-15,60)	23,33 (8,55-38,11)	-	<LOQ		
Ti (ng/ml)	0,33	<LOD	<LOD	5,16 (1,04-9,29)	-	<LOQ		
V (ng/ml)	0,01	<LOD	<LOD	<LOD	-	<LOD		
Biomarkers								
MDA (pg/ml)	72	74,53 (59,76-89,31)	66,50 (43,31-89,70)	150,12 (113,22-187,02)	-	61 (23-99)		
8-isoprostane (pg/ml)	1	<LOD	<LOD	<LOD	-	<LOD		
8-OHdg (pg/ml)	0,5	<LOD	<LOD	<LOD	-	<LOD		
					-			
Acetate (µM)	0,27	27,39 (17,43-37,36)	15,47 (8,48-22,47)	saliva contamination	-	14,9 (4,8-24,9)		
Propionate (µM)	0,07	7,24 (3,33-11,16)	4,04 (2,51-5,57)	saliva contamination	-	3,81 (1,97-5,64)		
Butyrate (µM)	0,58	0,75 (0,46-1,04)	0,54 (0,50-0,59)	saliva contamination	-	0,46 (0,31-0,61)		
Formate (µM)	0,12	1,98 (1,78-2,18)	2,17 (1,83-2,52)	saliva contamination	-	2,00 (1,57-2,43)		
Lactate (µM)	0,11	7,67 (5,48-9,87)	8,29 (5,91-10,67)	saliva contamination	-	5,74 (1,84-9,64)		
Pyruvate (µM)	0,16	<LOD	<LOD	saliva contamination	-	0,002 (-0,005-0,009)		
Nitrite NO2-(µM)	0,2	1,42 (1,26-1,58)	1,15 (1,02-1,29)	saliva contamination	-	1,25 (0,93-1,57)		
Nitrate NO3- (µM)	0,07	4,44 (1,96-6,91)	6,82 (0,82-12,82)	saliva contamination	-	3,09 (2,31-3,87)		
sum NOx (µM)	-		4.81 (3.80-5.82)					
NO_2^-/NO_3^-	-		0.40 (0.29-0.52)					

Parameter measured		Guide values	Literature
NTA	**LOD**		
Particles number concentration (10^6 #/mL)	26	-	No data available
Median particle diameter (nm)		-	No data available
Metal concentration			
Zn (ng/ml)	0,33	-	Zn concentration in healthy adults : 1,6 (0,50-22) ng/ml median - 25th-75th percentile [266]
Cu (ng/ml)	0,07	-	Cu concentration in healthy adults : 0,6 (0,30-1,80) ng/ml median - 25th-75th percentile [266] // 1,25 ng/ml [267]
Ni (ng/ml)	0,01	-	Ni concentration in healthy adults : 0,2-0,87 ng/ml [266]
Ba (ng/ml)	0,01	-	No data available
Mn (ng/ml)	0,01	-	Mn concentration in healthy adults : 0,1-0,57 ng/ml [266]
Cr (ng/ml)	0,01	-	Cr concentration in healthy adults : 0,25-0,28 ng/ml [268]
Co (ng/ml)	0,01	-	No data available
Sb (ng/ml)	0,01	-	No data available
Pb (ng/ml)	0,01	-	Pb concentration in healthy adults : 0,02-0,1 ng/ml [266]
Al (ng/ml)	1,63	-	Al concentration in healthy adults : 1,20 (0,60-2,35) ng/ml [266]
Fe (ng/ml)	0,3	-	Fe concentration in healthy adults : 1,20 (0,25-6,00) ng/ml [266]
Mo (ng/ml)	0,01	-	Mo concentration in healthy adults : 0,03 (0,01-0,08) ng/ml [266]
Si (ng/ml)	7	-	Si concentration in healthy adults : 19 (0,5-47) ng/ml [266]
Ti (ng/ml)	0,33		OEL-8h from inhalation - nanoparticulate titane 0,80µg/m3 or 4 µg/m3 in 15min (ANSES)
V (ng/ml)	0,01		No data available
Biomarkers			
MDA (pg/ml)	72	-	300-4930 pg/ml
8-isoprostane (pg/ml)	1	-	3- 85 pg/ml [269]
8-OHdG (pg/ml)	0,5	-	10-18 pg/ml [270]
		-	
Acetate (µM)	0,27	-	Acetate concentration in healthy adults : 18,80 (8,06-48,79) µM mean + IQR [271]
Propionate (µM)	0,07	-	Propionate concentration in healthy adults : 4,63 (2,14-29,77) µM mean + IQR [271]
Butyrate (µM)	0,58	-	Butyrate concentration in healthy adults : 0,81 (0,64-2,36) µM mean + IQR [271]
Formate (µM)	0,12	-	No data available
Lactate (µM)	0,11	-	Lactate concentration in healthy adults : 26,05 (8,60-112,09) µM mean + IQR [271]
Pyruvate (µM)	0,16	-	No data available
Nitrite NO2-(µM)	0,2	-	Nitrite concentration in healthy adults : 0,43 (0,30-1,86) µM mean + IQR [271]
Nitrate NO3- (µM)	0,07	-	Nitrate concentration in healthy adults : 0,93 (0,33-3,82) µM mean + IQR [271]
sum NOx (µM)	-	-	-
NO_2^-/NO_3^-	-	-	-

**Values between LOQ and LOQ are not changed, the values under LOD have not been taken into account. Missing limits are indicated by a dash*. Vx= number of volunteers.

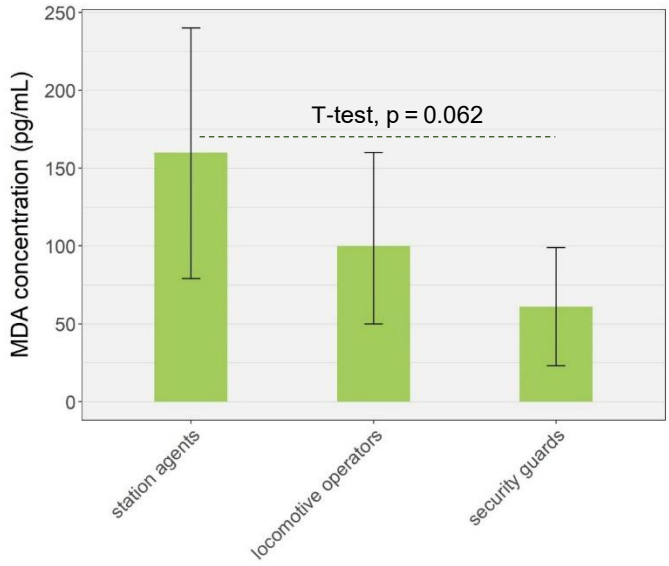


Figure 50. *Effect of the professional activity on the predicted EBC levels of MDA*

d. Averaged urine level of the considered variables

The average creatinine-adjusted metal concentrations in urine were in decreasing order: Si > Zn ≫ Mo > Ti > Cu > Ba ≈ Ni > Co, regardless of urine sampling time (Table 27). As for EBC, iron I not found consistently in urine. Cu, Mo, Ba, and Si concentrations were greater post-shift compared to pre-shift. Zn was the only metal that had greater pre-shift than post-shift urine concentrations for all workers. For other metals (Ti, Ni, Co), the pre- and post-shift variations were limited to one or two jobs. Most metal concentrations fell within the concentration range reported by guide values for general population, except for two values related to cobalt in volunteers 2 and 4.

Table 27. *Estimated geometric concentration or average concentration ± 95% confidence interval into bracket of the selected variables in urine samples collected during the two working weeks for the three professional categories. The concentrations are compared either to regulatory values when they exist, or to guide values in the literature.*

Parameter measured		Mean concentration (95% confidence interval)*				Predicted mean concentration (95% confidence interval) accorded to the model
		V1	V2	V3	**Shift**	Station agents
Metal concentration	**LOD**					
Si (µg/g creatinine)	133 ng/ml	6601,76 (5495,63-7707,89)	8675,73 (7453,67-9897,78)	9558,83 (8032,88-11084,77)	pre-shift	6786,36 (5901,40- 7671,32)
					post-shift	8819,21 (7502,73- 10135,70)
Zn (µg/g creatinine)	8,29 ng/ml	127,70 (115,45-139-96)	415,61 (363-467,81)	47,34 (39,72-54,95)	pre-shift	137,69 (54,25- 221,13)
					post-shift	114,2 (44,56- 183,83)
Ti (µg/g creatinine)	6,67 ng/ml	32,29 (27,8-36,77)	27,43 (22,64-32,22)	36,87 (25,33-48,40)	pre-shift	22,7 (4,91- 40,49)
					post-shift	36,69 (7,44- 65,95)
Mo (µg/g creatinine)	1,60 ng/ml	26,06 (20,94-31,17)	30,75 (25,34-36,15)	12,82 (11,06-14,58)	pre-shift	18,41 (12,08- 24,74)
					post-shift	22,27 (13,99- 30,55)
Cu (µg/g creatinine)	0,93 ng/ml	7,39 (7,01-7,76)	7,80 (7,14-8,46)	6,48 (5,68-7,27)	pre-shift	6,91 (5,47- 8,36)
					post-shift	6,97 (5,47- 8,47)
Ba (µg/g creatinine)	0,13 ng/ml	1,62 (1,29-1,95)	1,17 (1,00-1,35)	2,67 (1,76-3,57)	pre-shift	1,24 (0,47- 2,02)
					post-shift	1,93 (0,71- 3,16)
Ni (µg/g creatinine)	0,26 ng/ml	0,92 (0,65-1,19)	2,11 (1,81-2,40)	0,75 (0,70-0,80)	pre-shift	1,16 (0,54- 1,78)
					post-shift	0,92 (0,42- 1,42)
Co (µg/g creatinine)	0,07 ng/ml	0,45 (0,37-0,52)	**2,03 (1,76-2,30)**	0,27 (0,23-0,32)	pre-shift	0,64 (0,09- 1,20)
					post-shift	0,49 (0,06- 0,92)
Al (µg/g creatinine)	4,17 ng/ml	4,05 (1,40-6,70)	<LOD	<LOD	-	<LOQ
Cr (µg/g creatinine)	0,33 ng/ml	0,20 (0,17-0,23)	<LOD	<LOD	-	<LOQ
Fe(µg/g creatinine)	8,3 ng/ml	12,58 (10,70-14,47)	<LOD	19,71 (14,15-25,28)	-	<LOQ
Mn (µg/g creatinine)	0,08 ng/ml	0,08 (0,04-0,012)	<LOD	<LOD	-	<LOQ
Pb (µg/g creatinine)	0,47 ng/ml	0,31 (0,27-0,35)	<LOD	<LOD	-	<LOQ
Sb (µg/g creatinine)	0,1 ng/ml	<LOD	<LOD	<LOD	-	<LOD
V (µg/g creatinine)	0,1 ng/ml	0,07 (0,06-0,08)	<LOD	<LOD	-	<LOQ
Biomarkers						
1-hydroxypyrene (ng/mg creatinine)**	0,1 ng/ml	0,26 (0,20-0,32)	<LOD	0,12 (0,10-0,15)	-	not yet computed
MDA (ng/mg creatinine)	3,3 ng/ml	49,78 (43,26-56,30)	56,75 (39,40-74,11)	73,00 (41,20-104,80)	-	not yet computed
8-OHdg (ng/mg creatinine)**	0,3 ng/ml	3,69 (3,28-4,09)	1,91 (1,55-2,27)	2,79 (2,54-3,05)	-	not yet computed
8-isoprostane (ng/mg creatinine)**	0,05 ng/ml	0,57 (0,47-0,66)	0,19 (0,16-0,21)	0,24 (0,21-0,27)	-	not yet computed

Parameter measured		Mean concentration (95% confidence interval)*				Predicted mean concentration (95% confidence interval) accorded to the model	
		V4	V5	V6	**Shift**	Locomotive operators	
Metal concentration	**LOD**						
Si (µg/g creatinine)	133 ng/ml	4154,81 (3659,18-4650,45)	4166,70 (3609,59-4723,81)	4168,40 (3826,00-4510,79)	pre-shift	4233,52	(3666,52- 4800,53)
					post-shift	3863,69	(3327,66- 4399,72)
Zn (µg/g creatinine)	8,29 ng/ml	200,99 (188,34-213,64)	247,39 (215,57-279,22)	308,19 (288,65-327,73)	pre-shift	261,54	(102,90- 420,18)
					post-shift	232,95	(91,43- 374,48)
Ti (µg/g creatinine)	6,67 ng/ml	29,38 (24,48-34,27)	9,49 (6,12-12,85)	14,38 (8,83-19,92)	pre-shift	14,4	(3,06- 25,75)
					post-shift	12,5	(2,54- 22,46)
Mo (µg/g creatinine)	1,60 ng/ml	18,86 (14,91-22,81)	16,59 (11,78-21,41)	24,04 (19,29-28,79)	pre-shift	16,76	(10,91- 22,61)
					post-shift	17,73	(11,40- 24,06)
Cu (µg/g creatinine)	0,93 ng/ml	4,60 (4,30-4,91)	3,52 (3,07-3,96)	6,11 (5,52-6,70)	pre-shift	4,27	(3,37- 5,17)
					post-shift	4,91	(3,86- 5,95)
Ba (µg/g creatinine)	0,13 ng/ml	3,14 (2,69-3,59)	1,30 (0,36-2,23)	0,63 (0,49-0,78)	pre-shift	0,91	(0,34- 1,49)
					post-shift	1,13	(0,42- 1,84)
Ni (µg/g creatinine)	0,26 ng/ml	0,77 (0,68-0,86)	0,57 (0,43-0,71)	1,00 (0,74-1,25)	pre-shift	0,74	(0,34- 1,13)
					post-shift	0,69	(0,31- 1,06)
Co (µg/g creatinine)	0,07 ng/ml	**0,97 (0,80-1,15)**	0,19 (0,17-0,21)	0,11 (0,10-0,12)	pre-shift	0,24	(0,03- 0,44)
					post-shift	0,24	(0,03- 0,46)
Al (µg/g creatinine)	4,17 ng/ml	<LOD	<LOD	<LOD	-	<LOD	
Cr (µg/g creatinine)	0,33 ng/ml	<LOD	<LOD	0,24 (0,22-0,27)	-	<LOQ	
Fe(µg/g creatinine)	8,3 ng/ml	8,80 (7,53-10,08)	<LOD	<LOD	-	<LOQ	
Mn (µg/g creatinine)	0,08 ng/ml	<LOD	<LOD	<LOD	-	<LOD	
Pb (µg/g creatinine)	0,47 ng/ml	0,45 (0,40-0,51)	<LOD	<LOD	-	<LOQ	
Sb (µg/g creatinine)	0,1 ng/ml	<LOD	<LOD	<LOD	-	<LOD	
V (µg/g creatinine)	0,1 ng/ml	0,11 (0,09-0,12)	<LOD	0,09 (0,07-0,10)	-	<LOQ	
Biomarkers							
1-hydroxypyrene (ng/mg creatinine)**	0,1 ng/ml	0,09 (0,07-0,10)	<LOD	0,15 (0,12-0,18)	-	not yet computed	
MDA (ng/mg creatinine)	3,3 ng/ml	50,13 (27,47-72,80)	37,85 (31,84-43,86)	70,12 (38,40-101,84)	-	not yet computed	
8-OHdg (ng/mg creatinine)**	0,3 ng/ml	1,42 (1,17-1,68)	3,34 (2,98-3,71)	2,76 (2,37-3,15)	-	not yet computed	
8-isoprostane (ng/mg creatinine)**	0,05 ng/ml	0,15 (0,14-0,17)	0,13 (0,11-0,14)	0,27 (0,18-0,35)	-	not yet computed	

Parameter measured		Mean concentration (95% confidence interval)*				Predicted mean concentration (95% confidence interval) accorded to the model	
		V7	V8	V9	**Shift**	Security guards (V7-V8-V9)	
Metal concentration	**LOD**						
Si (µg/g creatinine)	133 ng/ml	5509,81 (4709,67-6309,95)	5764,75 (4116,16-7413,33)	6202,01 (5625,22-6778,81)	pre-shift	5089.95	(4409.88; 5770.02)
					post-shift	6014.52	(5221.72; 6807.31)
Zn (µg/g creatinine)	8,29 ng/ml	135,56 (112,00-159,12)	229,76 (189,06-270,46)	266,68 (243,70-289,67)	pre-shift	233.74	(91.97; 375.51)
					post-shift	161.36	(63.54; 259.18)
Ti (µg/g creatinine)	6,67 ng/ml	9,24 (7,44-11,04)	9,98 (6,08-13,87)	57,71 (54,68-60,74)	pre-shift	15.09	(3.18; 27.01)
					post-shift	11.20	(2.35; 20.06)
Mo (µg/g creatinine)	1,60 ng/ml	19,69 (15,19-24,19)	40,96 (27,63-54,29)	33,01 (26,18-39,84)	pre-shift	26.62	(17.34; 35.91)
					post-shift	25.06	(16.38; 33.73)
Cu (µg/g creatinine)	0,93 ng/ml	5,29 (4,90-5,69)	3,76 (3,28-4,25)	3,36 (2,95-3,76)	pre-shift	3.97	(3.14; 4.81)
					post-shift	3.92	(3.10; 4.74)
Ba (µg/g creatinine)	0,13 ng/ml	0,25 (0,20-0,30)	0,47 (0,31-0,63)	0,82 (0,69-0,95)	pre-shift	0.48	(0.18; 0.78)
					post-shift	0.35	(0.13; 0.57)
Ni (µg/g creatinine)	0,26 ng/ml	2,53 (2,30-2,76)	1,10 (0,50-1,69)	0,69 (0,58-0,79)	pre-shift	0.92	(0.43; 1.42)
					post-shift	1.28	(0.60; 1.97)
Co (µg/g creatinine)	0,07 ng/ml	0,19 (0,16-0,22)	0,11 (0,08-0,13)	0,12 (0,10-0,14)	pre-shift	0.11	(0.01; 0.21)
					post-shift	0.14	(0.02; 0.27)
Al (µg/g creatinine)	4,17 ng/ml	<LOD	<LOD	<LOD	-	<LOD	
Cr (µg/g creatinine)	0,33 ng/ml	<LOD	0,23 (0,19-0,28)	0,30 (0,26-0,34)	-	<LOQ	
Fe(µg/g creatinine)	8,3 ng/ml	<LOD	6,88 (5,11-8,65)	12,68 (12,07-12,29)	-	<LOQ	
Mn (µg/g creatinine)	0,08 ng/ml	<LOD	<LOD	<LOD	-	<LOD	
Pb (µg/g creatinine)	0,47 ng/ml	<LOD	0,49 (0,43-0,56)	<LOD	-	<LOQ	
Sb (µg/g creatinine)	0,1 ng/ml	<LOD	<LOD	<LOD	-	<LOD	
V (µg/g creatinine)	0,1 ng/ml	<LOD	0,07 (0,05-0,08)	0,06 (0,06-0,07)	-	<LOQ	
Biomarkers							
1-hydroxypyrene (ng/mg creatinine)**	0,1 ng/ml	0,13 (0,10-0,16)	0,15 (0,12-0,18)	0,14 (0,12-0,16)	-	not yet computed	
MDA (ng/mg creatinine)	3,3 ng/ml	79,64 (60,49-98,78)	63,56 (43,08-84,03)	69,09 (55,67-82,50)	-	not yet computed	
8-OHdg (ng/mg creatinine)**	0,3 ng/ml	4,01 (3,68-4,34)	2,81 (2,53-3,08)	2,77 (2,59-2,95)	-	not yet computed	
8-isoprostane (ng/mg creatinine)**	0,05 ng/ml	0,17 (0,16-0,18)	0,20 (0,17-0,22)	0,13 (0,12-0,15)	-	not yet computed	

Parameter measured		Regulatory values	Guide values	Literature
Metal concentration	**LOD**			
Si (µg/g creatinine)	133 ng/ml	-	No data available	
Zn (µg/g creatinine)	8,29 ng/ml	-	-	< 680 µg/g creatinine (95th percentile) [272]
Ti (µg/g creatinine)	6,67 ng/ml	-	-	in general population : Females : 32,69 -121,73 µg/g creatinine (mean -95th percentile), Males : 30,19-63,62 µg/g creatinine (mean - 95th percentile) [273]
Mo (µg/g creatinine)	1,60 ng/ml	-	< 114 µg/g creatinine (95th percentile) in general population	
Cu (µg/g creatinine)	0,93 ng/ml	-	-	< 12 µg/g creatinine (95th percentile) in general population [274]
Ba (µg/g creatinine)	0,13 ng/ml	-	-	< 6 µg/g creatinine (95th percentile) in general population [274]
Ni (µg/g creatinine)	0,26 ng/ml	-	-	< 3,38 µg/g creatinine (95th percentile) in general population [275]
Co (µg/g creatinine)	0,07 ng/ml	5 µg/g creatinine at post-shift and end of the week (ANSES)	For general population (non-exposed) Females: 1.5 µg/g creatinine; Males: 0.6 µg/g creatinine (ANSES)	-
Al (µg/g creatinine)	4,17 ng/ml	-	< 15 µg/g creatinine -general adult population	-
Cr (µg/g creatinine)	0,33 ng/ml	for Cr VI 1,8 µg/g creatinine ar the end of the week (ANSES)	-	Cr-based compounds : <0,54 µg/g creatinine (95ème percentile) [275]
Fe (µg/g creatinine)	8,3 ng/ml	-	No data available	
Mn (µg/g creatinine)	0,08 ng/ml	-	< 0,5 µg/g creatinine (95th percentile) in general population (National Health and Nutrition Examination Survey, USA)	-
Pb (µg/g creatinine)	0,47 ng/ml	-	< 1,1 µg/g creatinine in general population (National Health and Nutrition Examination Survey, USA)	-
Sb (µg/g creatinine)	0,1 ng/ml	-	-	< 0,25 µg/g creatinine (95th percentile) in general population (National Health and Nutrition Examination Survey, USA) [275]
V (µg/g creatinine)	0,1 ng/ml	-	-	< 1,12 µg/g creatinine (95th percentile) in general population [272]
Biomarkers				
1-hydroxypyrene (ng/mg creatinine)**	0,1 ng/ml		< 0,3 ng/mg creatinine (95ème percentile) at post-shift for non-occupationally exposed adult people (National Health and Nutrition Examination Survey, USA)	
MDA (ng/mg creatinine)	3,3 ng/ml	-	-	100 (70-120) ng/mg creatinine and mean and 95% percentile confidence interval
8-OHdG (ng/mg creatinine)**	0,3 ng/ml	-	-	concentration in healthy adults: 3.9 (3-5,5) ng/mg creatinine - mean and IQR [276]
8-isoprostane (ng/mg creatinine)**	0,05 ng/ml	-	-	concentration in healthy adults: 0,18-0,40 ng/mg creatinine - mean and IQR [277]

*Values between LOQ and LOQ are not changed, the values under LOD have not been taken into account. **values below the LOQ had an assigned value of LOQ/root(2). Vx= number of volunteers. Missing limits are indicated by a dash.

e. Exploratory correlation analysis.

To understand if there was a relationship between parameters characterizing exposure and effect biomarkers, we performed an exploratory pairwise correlation analysis. Concerning MDA in EBC, no correlation was observed between MDA and metals in EBC or between MDA and EBC's sub-micrometer particles. Significant positive correlation was nevertheless found between MDA and different anions measured in EBC samples (Table 28). A low but statistically significant positive correlation was observed between acetate/ NO_2^-/NO_3^- and MDA. Formate was positively and strongly associated with MDA.

Table 28. *Correlation between EBC levels of MDA and the different anions*

	Formate [µmol/L]	Acetate [µmol/L]	Lactate [µmol/L]	∑ NO_x [µmol/L]	NO_2^-/NO_3^-
MDA [pg/mL]	**0.53 (0.001)**	**0.29 (0.001)**	0.12 (0.169)	0.06 (0.468)	**0.29 (0.001)**

Pearson correlation coefficients, with p-value between brackets. Bold values indicate a statistically significant correlation (p<0.05).

Only five variables for anions were considered in this analysis because propionate and butyrate were both strongly correlated with acetate, and pyruvate levels were very low and near the LOD

We also attempted to determine whether the change in concentration of MDA during a work-shift (the difference between end of shift (e) and before shift (b) could be due to the PM exposure, either measured the same day (lag 0), one day before (lag 1). The developed statistical model [265] indicated a positive and strong association between the variation of MDA level during the working day and exposure to PM_{10} encountered the day before (p=0.05; lag 1, Table 29).

Table 29. *Association between personal exposures and MDA at lag 0 or 24 hours after exposure (lag 1)*

Personal exposure	MDA_e/MDA_b Lag 0	MDA_e/MDA_b Lag 1
Particle number concentration [#/cm^3]	-0.06±0.22 (0.77)	0.02±0.19 (0.94)
UFP size [nm]	-0.55±0.68 (0.42)	-0.97±0.74 (0.19)
Lung deposited surface area [$\mu m^2/cm^3$]	-0.12±0.25 (0.62)	-0.08±0.22 (0.74)
$PM_{2.5}$ [$\mu g/m^3$]	-0.03±0.20 (0.89)	0.46±0.30 (0.13)
PM_4 [$\mu g/m^3$]	-0.66±0.48 (0.17)	-0.08±0.51 (0.88)
PM_{10} [$\mu g/m^3$]	-0.06±0.23 (0.81)	**0.52±0.26 (0.05)**

For each pair exposure-MDA, the association was evaluated using linear regression models with the (log-transformed) daily evolution of MDA (ratio post-shift/pre-shift) as dependent variable, the personal exposure as independent predictor variable, and BMI as adjustment variable to control for confounding/effect modification. Bold values correspond to coefficients statistically significantly different from zero. Indice $_e$ corresponds to post-shift MDA level whereas indice $_b$ corresponds to pre-shift MDA level.

f. OPEA results

We did not find any difference in the OPEA values between different subways occupations and the days of the week (corrected by the job effect and ambient OPEA) (Figure 51 and Figure 52). The developed statistical model also indicated no association between the variation of OPEA values during the working day and exposure metrics exposure except for Barium in the PM_{10} fraction where it had a significant negative correlation (p=0.028).

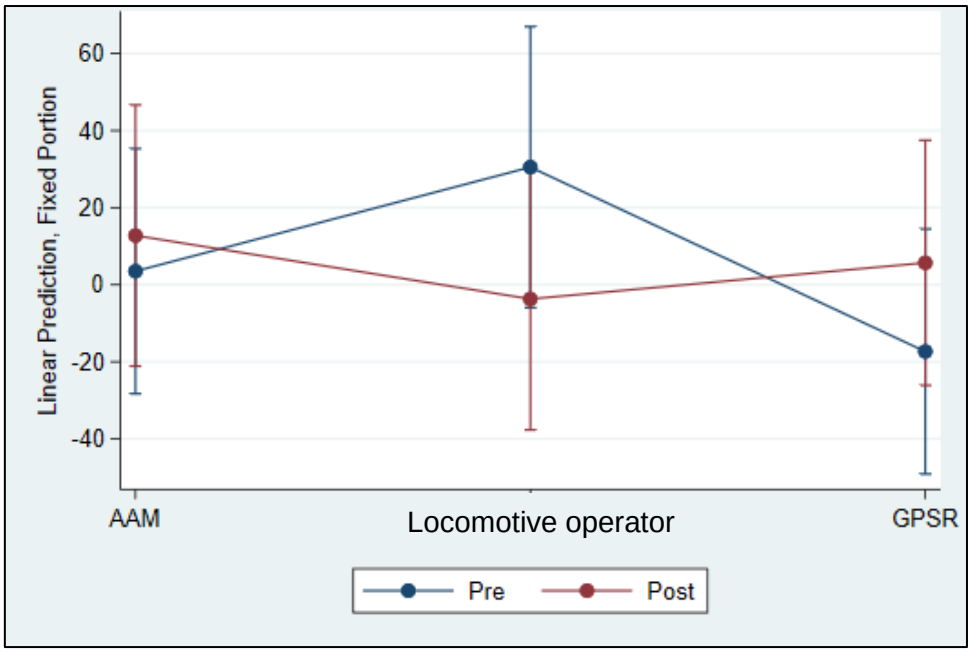


Figure 51. *Predicted values for alveolar OPEA (pmol/L)*

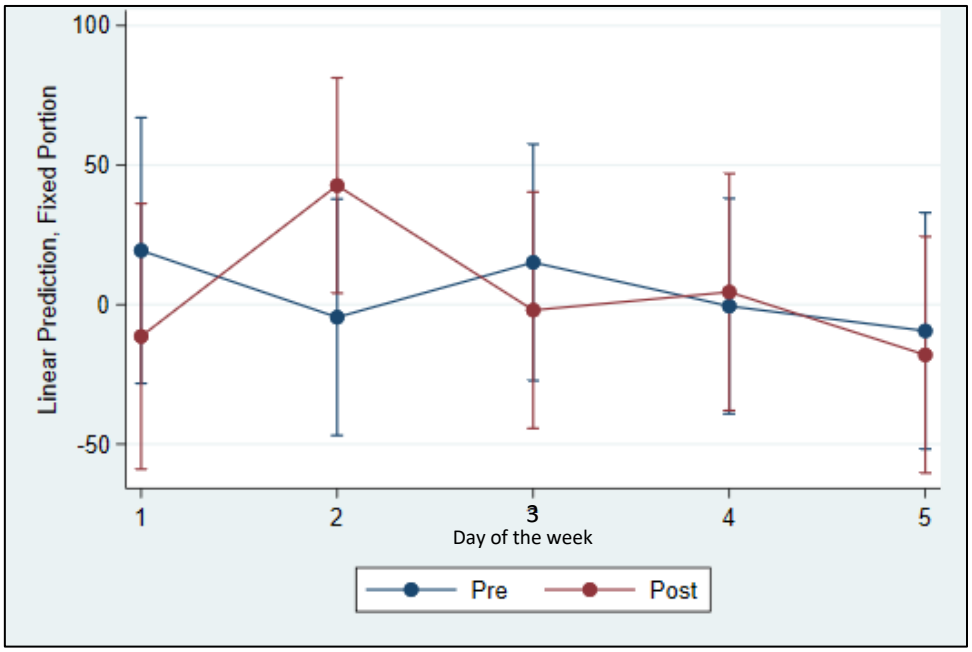


Figure 52. *Ambient corrected alveolar OPEA (pmol/L)*

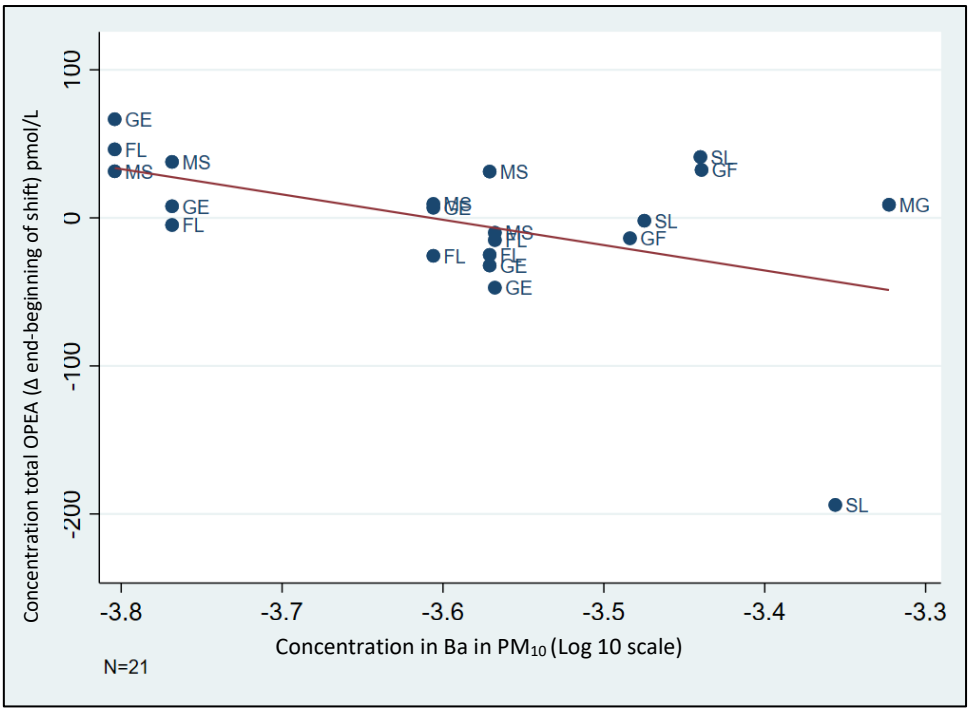


Figure 53. *Ba concentration in PM10 as a function of total OPEA during the day (pmol/L)*

4. My contribution to the Field study

I was responsible of the compliance of good sampling practices and managed medical RATP staff to perform specific sampling and environmental measurements on a minimum of 30 workers per day.

The data were analyzed at the end of 2021 but it is still too preliminary to display the first findings, the data being not yet in the format required for statistical analysis. First results will be provided in the course of 2022

REFERENCE VALUES FOUND IN THE LITERATURE

I. Context

The population-based reference interval is the most widely used tool for interpretation of individual clinical test results. It is essential to identify individuals with abnormal (increase or decrease) observed values for example in case of COPD, providing it is accurate. Indeed, if the relevant reference interval is invalid or deficient in some way, all efforts directed at ensuring analytically precise and accurate test results will be to a greater or lesser extent useless.

The terminology relevant to the reference interval establishment was defined by the International Federation of Clinical Chemistry (IFCC) and the most important terms for understanding the procedure of reference interval estimation are defined below.

The value (test result) obtained by observation or measurement of a particular quantity on an individual belonging to a reference sample group is called **Reference value**.

The **reference sample group** is an adequate number of reference individuals (i.e., an individual selected for comparison using defined criteria) taken to represent the reference population. Ideally, the reference individuals should be randomly drawn from the reference population. The **reference population** is a hypothetical entity, which consists of all possible reference individuals. The statistical distribution of reference values is called **Reference distribution**. The reference distribution enables derivation of a **Reference limit** so that a stated fraction of the reference values is less than or equal to, or more than or equal to the respective upper or lower limit. Usually, the two reference limits are the lower and upper limits of the 95% confidence interval around the mean value estimated in the reference distribution.

The interval between and including two reference limits is called **Reference interval**. This term replaced the term “reference range”. The latter was rejected because statistically a range is the difference between the highest and lowest value in a number set (e.g., interquartile range) and is a single value.

Finally, the value of a particular marker quantity obtained by a measurement is called **Observed value**. It corresponds to the individual test result and can be compared with reference values, reference distributions, reference limits or reference intervals.

Reliable and accurate reference intervals for laboratory analyses are an integral part of the process of correct interpretation of clinical laboratory test results [278]. Reference intervals helps the clinician in interpreting test results and are complementary to clinical decision limits. Indeed, while the latter are associated with a significantly higher risk of adverse clinical outcomes or are diagnostic for the presence of a specific disease, the reference intervals describe the typical distribution of results characteristic of a healthy reference population [279].

In the late eighties, the term 'normal values', considered inadequate and even partially incorrect was substituted with the term "reference values". The IFCC released a series of recommendations to encourage clinical chemistry laboratories to follow defined procedures to produce its own reference values. However, the most significant step in the development of reference intervals was the release of the C28-A3 guideline, by the Clinical and Laboratory Standards Institute (CLSI) and IFCC in 2008 [280]. This guideline entitled 'Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory' is still in current use and defines all the necessary steps in reference interval establishment, including the selection of reference individuals, pre-analytical and analytical considerations, and estimation of reference values and the reference interval.

The process of obtaining reference values in a healthy population is complex. The study samples must be representative of the general healthy population and of sufficient size. Data on the different variables must be reported, to satisfy the statistical power of the analysis.

As several studies reported the OS biomarkers levels in the EBC of healthy adults (reference population) we proposed to use meta-analyses to summarize the reference values for the biomarkers used in this project.

For this, I have attempted to develop a comprehensive protocol to systematically study the inter- and intra-individual variability of oxidative stress biomarkers collected from non-invasive matrices such as urine, EBC and exhaled air as a result of environmental and occupational exposure in healthy volunteers. The biomarkers chosen in this study are 8-OHdG, 8-isoprostane, MDA and H_2O_2. The protocol has been published on the International Prospective Register of Systematic reviews website with the registration number CRD 42020146623. The types of variability that I studied were stability during the day (circadian rhythm), the influence of fasting or food (food supplements e.g., vitamins), sport, smoking, age, sex, body type, ethnicity, genetics as well as the collection and analysis equipment.

In addition to this variability, I descriptively assessed the intrinsic quality of the biomarker by considering the inter-study reproducibility. The profile of this biomarker candidate was then evaluated for its suitability for use in different exposure conditions and other intra-inter-

individual variables, to be integrated into occupational biological monitoring. For this thesis work, I will present the method that I used for 8-OHdG, 8-isoprostane in the EBC matrix and present their respective outcomes. For MDA, the meta-analysis is not submitted yet, so the results cannot be provided in the thesis.

II. Overview of the 8-OHdG results

The lack of standardization in the use of EBC to measure 8-OHdG in a clinical setting is a major concern. Multiple collection devices are available and individual investigators decide on the device as well as the protocol in terms of sample collection and analysis. This leads far too much variability in collection times, temperatures and conditions of analysis among these studies. Until methods are standardized, it will be difficult to reliably compare information between different studies and to establish reference values for 8-OHdG. If these methods were to be improved, this would notably be achieved through the establishment of standardized protocols and consistent guidelines between research laboratories. This methodological requirement is still essential for the clinical development of the oxidative stress biomarkers approach.

The work on 8-OHdG was the subject of a publication in **Tox letters** in March 2020 with the heading: **Reference ranges of oxidative stress biomarkers selected for non-invasive biological surveillance of nanotechnology workers: Study protocol and meta-analysis results for 8-OHdG in exhaled breath condensate.**
This project was also the subject of a **poster** for 11th International Symposium on Biological Monitoring in Occupational and Environmental Health (ISBM-11) in Leuven- Belgium.

1. Methods

a. Literature search

Searches were conducted for literature published since journal inception and up to March 26 2019 in the following bibliographic electronic databases: The Cochrane Central Register of controlled Trials (CENTRAL, Cochrane Library), EMBASE, PubMed, and Web of Science. The search strategy was adapted specifically for each electronic database used. To enlarge the documents search and ensure a systematic review as exhaustive as possible, a combination of the MeSH (Medical Subject Headings) terms from the PubMed database Emtree terms from the EMBASE database and free text words was used for each of biomarkers considered. This strategy allowed expanding this review to additional biomarkers by adding terms corresponding to them. The complete search strategy (exemplified by the search in PubMED and EMBASE databases) is available as a supplementary digital content (https://www.doi.org/10.16909/dataset/17). Original research studies in healthy human participants (aged 18+, no known disease) measuring the biomarkers in EBC written in English or French were included. The studies without quantitative data, non-human, in -vitro studies, reviews, correspondence, conference papers, expert opinions and editorials, as well as abstracts without full text were excluded. Furthermore, we also excluded studies where EBC collection device failed to meet American Thoracic Society and European Respiratory Society methodological recommendations [179].

Two reviewers (MH and YS) independently performed a first screening of titles and abstracts retrieved during the searches, using Rayyan software [281]. Abstracts with insufficient information with regard to the inclusion and exclusion criteria were downloaded in the EndNote software for a full-text screening. The same reviewers independently assessed each article. Disagreements on the inclusion/exclusion of studies between two reviewers were discussed and solved by consensus; when necessary a third reviewer (IGC) was consulted to reach consensus.

b. Data extraction

To gather the essential information according to our research objectives, we developed a standardized data extraction form, which was pre-tested by two reviewers (MH and MG) and validated by a statistician (PW). The information as follows: first author name, publication time, study type, analytic method, the sample time, the number of participants, the gender, the mean age, the mean BMI, their smoking status, season, occupation, pregnancy, diet,

vitamin, exercise, outcomes, was considered of interest and extracted by two independent reviewers (YS and MH). When data on several subgroups were available in a given paper, all subgroup-specific data were extracted. In a second round, we excluded all subgroups selected based on a disease status (e.g., diabetics) and all subgroups selected based on an exposure status (e.g., welders). If data on the same population was reported at different moments (e.g., different seasons), only the data at inclusion were included. Finally, in a third round, duplicate data were excluded (e.g., the same control population was reported in more than one study). All quantitative data extracted were cross-checked by a statistician.

c. Quality assessment

Specifically for purpose of this review, we developed a checklist of quality assessment criteria, which was reviewed by the experts in epidemiology, biostatistics, toxicology, biomonitoring, chemistry, and pharmacology. This checklist included four domains: (i) quality of the study sample, (ii) quality of study design and risk of bias, (iii) technical and analytical quality (i.e., quality of biological sample collection and conservation and of the laboratory analyses), and (iv) quality of the data processing, analysis and result reporting. Each domain can be assessed separately, based on a number of objective criteria (Table 30), and graded by assigning a discreet sub-score value. The resulting sub-scores values can be further summarized in a final score for each study, as recommended in the GRADE guidelines [282]. The total quality scores ranged between 9 and 27. Quality scores lower or equal to 13 corresponded to a low quality of evidence; scores between 14 and 19 to a moderate quality of evidence, and scores higher than 20 to a high quality of evidence. The quality assessments of the included studies was performed by two reviewers (YS, MH) independently, the discrepancies were solved through discussion and the quality assessment by a third reviewer (IGC).

Table 30. *Quality appraisal and scoring criteria*

Criteria for quality appraisal	Instructions for assessment	Number of points for Quality scoring		
		1 point	2 points	3 points
I. Study sample quality				
I.1. Representativeness of the study sample	The sample design and sample construction strategy should be clearly described in the article. If not, please consider the inclusion and exclusion criteria	Convenience sample	Consecutive sample	Probability sample (simple random sample, systematic sample, stratified random sample or cluster sample)
I.2. Between group comparability and potential for selection bias	To appraise this criteria, please consider the inclusion and exclusion criteria in each of the groups considered, depending on the study design (exposed/ unexposed or healthy/ ill) and the comparability of the groups finally selected in terms of all criteria except the exposure or the health outcome (statistical tests or sample descriptive statistics)	The cases and controls or exposed and unexposed participants are selected from the different settings and present different social, demographical, and biological characteristics (p-values < 0.05 or not reported, or not assessed)	An intermediate situation, where the groups are somehow comparable, because of the same criteria or the target/ accessible population used for the participant selection, and somehow comparable descriptive statistics between groups (no statistically significant difference for most of the characteristics between groups	The cases and controls or exposed and unexposed participants are selected from the same population, based on the same inclusion/ exclusion criteria and participation rate is similar in the both groups. Moreover, the statistical comparison of the groups did not reveal any statistically significant difference in terms of participant characteristics other than the exposure or the outcome, depending on the study design
I.3. Sample size and statistical power	To appraise this criteria, please consider the number of subjects in reference and in each group should be considered for the appraisal of this criteria	< 20	20–50	≥ 50
II. Study design quality				
II.1. Potential of confounding bias	To appraise this criteria, please consider how the authors address the potential confound for at least three following factors: Age-, Sex-, Smoking status (1 = significantly different or no data collected; 2 = not statistically different; 3 = matched or reported by risk factor group	The reported results are significantly different with respect to the age, sex and smoking status or no data on them were collected	The reported results are not significantly different with respect to the age, sex and smoking status	The cases and controls were matched on these factors, or the results are reported by risk factor group (as result of the analyses stratified by age, sex and smoking status)
II.2.a Potential for misclassification bias on the exposure	To appraise this criteria, please consider how measurement of the exposure was done and which data were used for assessing cumulative exposure. If the study design is based on the healthy/ ill participants, please address the criteria II.2b	Assessed by researcher, without individual data, or with only qualitative individual data (questionnaires)	Assessed by the research team including hygienists air measurements and individual data on duration of employment	Assessed based on biological monitoring measurements and individual work history data
II.2.b Potential for misclassification bias on the outcome	To appraise this criteria, please consider how the diagnosis of disease was done	The cases were identified using existent medical records	The cases were examined by a study physician and confirmed as such	All study subjects were examined by the expert physician, and the diagnosis of each case was confirmed using the reference diagnostic procedure
III. Analytical/ technical quality	To appraise this criteria, please consider how thoroughly the laboratory analysis were conducted, in particular whether the author described the comparability of assessment methods if there is more than one group, the solvents used, the detector parameters (e.g., masse, UV-V), the internal or external calibration, and in case of internal calibration, the name/ RT of the compounds (except for immuno-essays), the LOD, LOQ, linearity, specificity, precision, accuracy, recovery rate, ,description of matrix effect, contamination, and criteria for acceptance of the method	Only LOD and LOQ were specified	Only 1/3 - small half of criteria were specified	More than half or 2/3 of the criteria were specified
III.1. Quality of laboratory analysis				
III.2 Measurements by mass spectrometry	Please assign the number of points accordingly to the specifications provided	Only succinct commentary of the method used was provided	The author followed and reporting the recommended guidelines used for their measurements	Full description of the validation of the method and guideline used, transitions, quantifications ions, confirmations ions, retention time of compounds, in case of internal calibration, the name/ RT of the compounds performed was reported
III.3. Measurements by ELISA or other techniques	Please assign the number of points accordingly to the specifications provided	Only succinct commentary of the method used was provided	The author set up their own technique and reported their validated analytical performance	The authors used a commercialized kit recognized as reference used
IV. Quality of the data processing, analysis and result reporting	To appraise this criteria, please consider how thoroughly the statistical methods were described, including the methods used to examine subgroups, confoundings, effect modifications and interactions, how missing data were addressed, and how the statistical significance was assessed. Please consider if the confidence intervals were given for the main results	Only succinct commentary of the method used was provided	Only some of criteria were specified and only p-values were reported for the significance of the results	More than 2/3 of the criteria were specified and confidence intervals were reported for the main results

d. Statistical analysis

- As primary research outcome, we analyzed the baseline values of biomarkers measured in original studies of healthy non-smoking adults in view of establishing the reference ranges using meta-analysis. Values of biomarkers selected, in particular urinary biomarkers, were generally log-normally distributed [283], therefore for EBC, we also computed geometric means (GM) and geometric standard deviations (GSD) as the basis of the meta-analysis. GM and GSD computing accounted for the heterogeneity of original data reported. If the data were reported in terms of arithmetic means (AM) and standard deviations (SD), the arithmetic mean and the standard deviation of the log-transformed measurement of the biomarker was calculated as: $muL = \ln(GM)$ and $sdL = \ln(GSD)$, respectively.

If the variability parameter was given as the standard error of the mean (SEM), we first computed SD as a product of SEM and of square root of the number of subjects on which the SEM computed: $SD = SEM \,.\sqrt{N}$.

Then, $muL = \ln(AM) - 0.5\ln\left(1 + \frac{SD^2}{AM^2}\right) and\ sdL = \sqrt{\ln\left(1 + \frac{SD^2}{AM^2}\right)}$

- If the data were reported in terms of GM and 95%CI, we assume that the lower (LCL) and upper (UCL) confidence limits correspond respectively to

$LCL = \exp\left(\mathrm{muL} - 1.96\frac{\mathrm{sdL}}{\sqrt{N}}\right)$ and $UCL = \exp\left(\mathrm{muL} + 1.96\frac{\mathrm{sdL}}{\sqrt{N}}\right)$. Thus,

$$\mathrm{muL} = (\ln(\mathrm{LCL}) + \ln(\mathrm{UCL}))/2 \text{ and } sdL = (\ln(\mathrm{UCL}) - \ln(\mathrm{LCL})).\sqrt{N}/(\Phi(0.975) - (\Phi(0.025)),$$

where Φ is the cumulative distribution of the standard normal distribution.

- If the data were reported in terms of AM and 95% CI, we assume that the lower (LCL) and upper (UCL) confidence limits correspond respectively to

$LCL = AM - 1.96\frac{SD}{\sqrt{N}})$ and $UCL = AM + 1.96\frac{SD}{\sqrt{N}})$.

Thus, $\mathrm{AM} = \frac{\mathrm{LCL}+\mathrm{UCL}}{2}$ and $SD = (\mathrm{UCL} - \mathrm{LCL}).\sqrt{N}/(\Phi(0.975) - \left(\Phi(0.025)\right)$

From AM and SD, we applied the formulas cited above.

- If the data were reported in terms of quartiles (denoted Q1 and Q3 and/or median, we applied following formulas:

$\mathrm{muL} = (\ln(Q1) + \ln(\mathrm{Q3}))/2$ and $sdL = (\ln(\mathrm{Q3}) - \ln(\mathrm{Q1}))/(\Phi(0.75) - (\Phi(0.25)),$

with $\mathrm{muL} = \ln(median)$, which allowed an internal check of the statistics.

When neither SD, GSD, IQR nor IC were reported, making it impossible to compute standard errors on the geometric (or arithmetic scale), we excluded the studies from the meta-analysis. Before computing GM and GSD, we converted all data to the same units, (pg/mL for 8-OHdG in EBC). The analytical methods used were stratified by main quantification methods into immunochemical techniques and chemical analytical methods (in these papers liquid—chromatography coupled to mass spectrometry in tandem- LC-ESI-MS/MS, and ELISA) and data analyzed separately. For each biomarker of interest, other independent variables may be considered depending on the number of study groups and the available information in the studies.

According to standard practice in meta-analysis [284], the first step is to represent the data as forest plots including the I-square that estimates the percentage of the between-study heterogeneity. If the latter is very large, this means that the between-study heterogeneity is much larger than the between-subject heterogeneity and any attempt of obtaining a reference value for individual subjects will not be valid.

We used STATA, version 16 software for data management and statistical analyses. 8-OHdG measured in EBC was the first biomarker of interest we analyzed according to this protocol.

2. Results

a. Study selection

The application of the literature research string in four databases resulted in 19421 records (Figure 54). After removal of duplicates and selection of the studies where EBC was collected for analyses, 893 records remained. From these records, 589 were excluded based on the title and abstract reading. Among the remaining 304 records, 17 included 8-OHdG measurements in EBC. After reading these studies, eight were included into the narrative description and six into quantitative synthesis. The study by [285] was excluded because only the arithmetic mean was reported - no variability (SD GSD, IC IQR) indicated. The control group in [231] and in [286] was identical and was therefore not included twice. The other study- sub-groups presented in Pelclova articles were groups with various occupational exposures and were therefore not included.

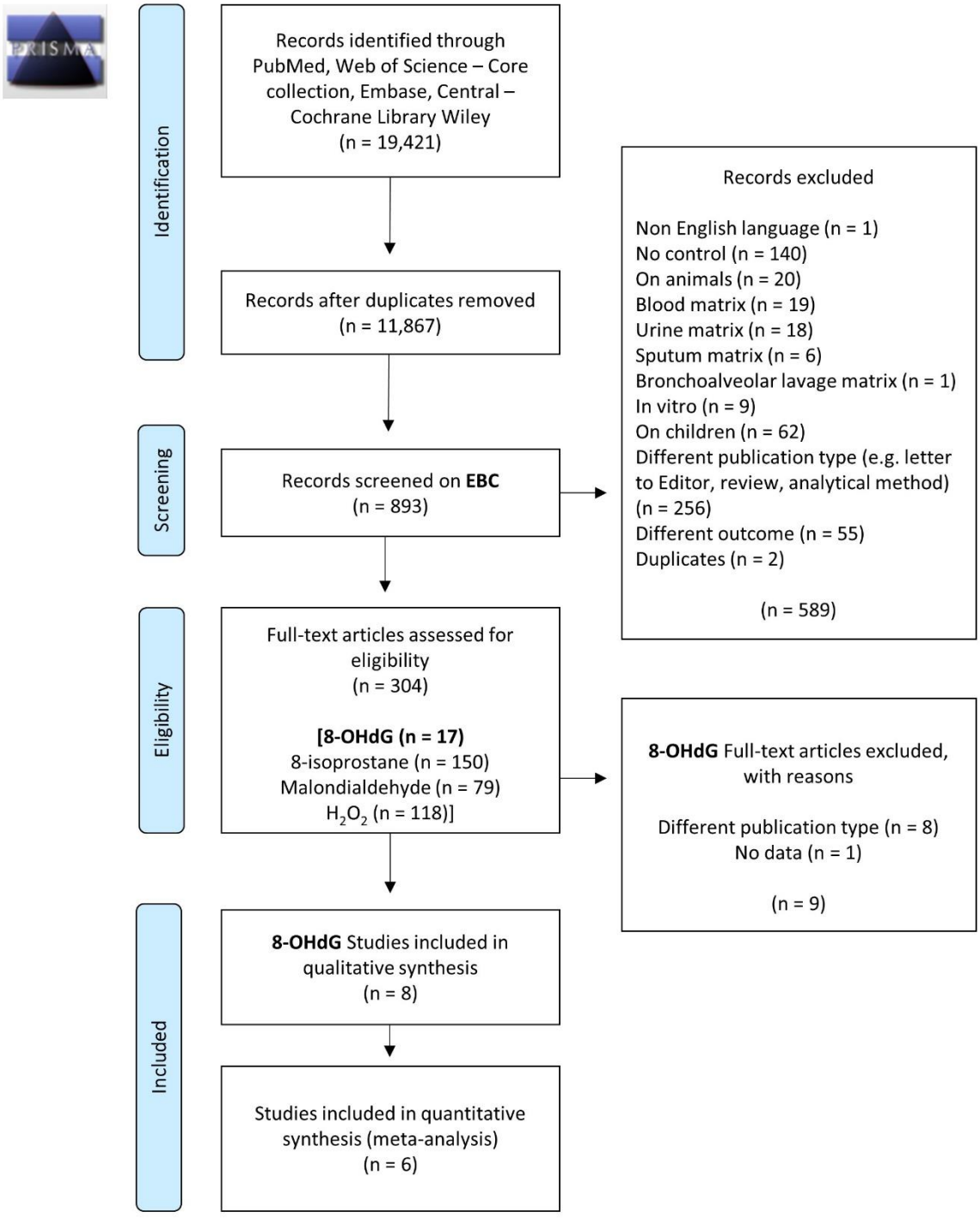


Figure 54. *Flow-chart of study selection*

b. Descriptive results

Among the eight studies included into the systematic review, five (62.5%) were of moderate quality and three of low quality of evidence (Table 31). Among the six studies included in the meta-analyses five were analyzed chemically and one by immunochemical methods. Table 31 summarizes all the included studies and provides detailed information on their respective quality, including sub-score values for each of four domains considered.

Table 31. *Summary of studies included into systematic review - meta-analysis*

	Ref 1 *	Ref 2 *	Ref 3
Author, year, country	Daniela Pelclova, 2012, Czech Republic.	Daniela Pelclova, 2016, Czech Republic.	Daniela Pelclova, 2016, Czech Republic.
Title, reference	Markers of oxidative stress are elevated in workers exposed to nanoparticles. Conference proceedings	Markers of oxidative damage of nucleic acids and proteins among workers exposed to TiO2 (nano)particles. Occup. Env. Med, 2016, 73(2) p 110-118	Oxidative stress markers are elevated in exhaled breath condensate of workers exposed to nanoparticles during iron oxide pigment production. J Breath Res, 2016,10(1) 016004.doi:10.1088/1752-7155/10/016004
The objective of the study	To study the possible adverse health-effects in workers exposed to TiO_2 aerosol through non-invasive method such as the analysis of markers of oxidative stress and inflammation in EBC and the measurement of FeNO.	To non-invasively measure and evaluate the markers of oxidation of nucleic acids and proteins in the EBC of workers and control subjects.	To evaluate markers of oxidative stress in the EBC of workers exposed to iron oxide nanoparticles during iron oxide pigment production and their association with workplace environments.
The short description of the exposed/sick sub-population (who are included, participant rate and number of participants)	20 workers exposed to TiO_2 aerosol (males, 11 smokers, 9 non-smokers).	36 workers inclusion criteria: males; the workers had to be working with TiO_2 for at least 6 months. Exclusion criteria for all subjects were: history of tuberculosis, myocarditis, congenital heart disease, lung cancer and recent fever and/or inflammation.	14 workers (males, 43 % smokers). Their mean length of exposure to iron oxides was 10 ± 4 years.
The short description of controls sub-population (who are included, participant rate and number of participants)	19 controls (males, 11 smokers, 8 non-smokers).	45 controls had comparable characteristics to the workers. These men were not employed in the factory; they worked as healthcare personnel and technical staff and did not handle nanomaterial or dusts/ aerosols.	14 males (50% smokers), who were not employed in this factory and were not exposed occupationally to dust or other health risks (safety inspectors and office workers).
Method of 8-OHdG measurement (when EBC collected…)	Pre-shift and post-shift EBC samples collected using Ecoscreen Turbo (DECCS, Jaeger, Germany). In the workers the measurements were performed both before and after 8 h shifts. identical breathing volume (120 liters) was maintained in all subjects; nose breathing was eliminated by nose-clips.	EBC samples were collected using an Ecoscreen Turbo (DECCS, Jaeger, Germany). All subjects breathed tidally for 15 min through a mouthpiece connected to the condenser (−20 °C) while wearing a nose-clip. The samples were immediately frozen and stored at − 80°C until analysis.	EBC samples were collected using Ecoscreen Turbo (DECCS, Jaeger, Germany). All subjects breathed tidally for about 15 min while wearing a nose-clip. Time of collection was about 15 min. All samples were immediately frozen and stored at −80 °C.
Short summary of the findings	This pilot study suggests the possibility of biological effects of chronic TiO_2 exposure to aerosol particles containing coarse, fine and nano-sized fractions. The results indicate that oxidative stress and elevated levels of oxidatively altered biomolecules are important intermediate endpoints that may be useful markers in hazard characterization of particulates	The concentration of titanium in EBC may serve as a direct exposure marker in workers producing TiO_2 pigment; the markers of oxidative stress reflect the local biological effect of (nano)TiO_2 in the respiratory tract of the exposed workers.	The findings suggest the adverse effects of nano iron oxide aerosol exposure and support the utility of oxidative stress biomarkers in EBC. Using the precautionary principle, all exposed employees, including research workers who are directly exposed to iron oxide nanoparticles should be monitored for potential side effects.
Quality sub-scores by domain			
I. Study sample quality	5	6	4
II. Study design quality	5	6	4
III. Analytical/ technical quality	2	3	2
IV. Quality of the data processing, analysis and result reporting	3	3	3
Final score *(sum of the points assigned to each of above-cited criteria, the highest score is 27 (3 points assigned to each of 9 criteria)*	15	18	13
Quality level (< or = 13 = Low; 14 to 19 = Moderate; > 20 = High)	Moderate	Moderate	Low

*studies included in the meta-analysis.

	Ref 4 *	Ref 5 *	Ref 6
Author, year, country	Daniela Pelclova, 2016, Czech Republic.	Kamila Syslova, 2010, Czech Republic.	Einat Fireman Klein, 2019, Israel.
Title, reference	Markers of nucleic acids and proteins oxidation among office workers exposed to air pollutants including (nano)TiO2 particles. Neuro Endocrinol Lett, 2016. 37 (Suppl 1) p 13-16	LC-ESI MS/MS method for oxidative stress multimarker screening in the exhaled breath condensate of asbestosis/ silicosis patients. J Breath Res, 2010. 4(1): p. 017104.	Ultrafine particles in airways: a novel marker of COPD exacerbation risk and inflammatory status. Int J Chron Obstruct Pulmon Dis, 2019. **14**: p. 557-564.
The objective of the study	To study a panel of biomarkers of nucleic acids and proteins oxidation in office workers exposed to TiO_2 for a short period of the shift during the control of production. The workers were studied using identical methods.	A highly selective and sensitive method is presented for the quantification of 8-iso-PGF2α, o-Tyr and 8-OHdG in EBC as significant biomarkers of oxidative stress in vivo. The method was tested on real clinical samples collected from patients diagnosed with asbestosis, silicosis and on the control group of healthy subjects.	To evaluate the exhaled breath condensate (EBC)- UFP content as a reflection of inflammation and oxidative stress status in COPD patients and as an exacerbation risk marker.
The short description of the exposed/sick sub-population (who are included, participant rate and number of participants)	22 office employees occupationally exposed to TiO_2 during their visit in the production workshops for average 14 ± 9 min/day.	10 patients(male, non-smokers) with occupational lung diseases (either silica- or asbestos-induced disorders due to mean 22 ± 6 years of occupational exposure to silica or asbestos). The subjects were treated for hypertension (20 %) and hyperlipidaemia (40 %).	58 COPD patients who were attending an outpatient clinic, "The Center for Treatment of Lung Diseases" (Hagefen Clinic). Patients were clinically stable at the time of their visit to the clinic, and there had been no exacerbation of their COPD within the previous 4 weeks.
The short description of controls sub-population (who are included, participant rate and number of participants)	14 control subjects not employed in the factory were examined; they worked in the offices as healthcare personnel and technical staff.	10 subjects(male, non-smokers) without any occupational exposure to fibrogenic dusts. The subjects were treated for hypertension (20%) and hyperlipidaemia (40%).	40 smokers and non-smokers with no lung diseases consisting of hospitalized patients and the health care staff from Bnai Zion Medical Center. Those who had been hospitalized for any infectious or inflammatory conditions were excluded.
Method of 8-OHdG measurement (when EBC collected…)	The EBC samples were collected using the Ecoscreen Turbo (DECCS, Jaeger, Germany). All subjects breathed tidally for 15 minutes while wearing a nose-clip.	The commercially available condenser EcoScreen (Jaeger, Germany) was used to collect EBC samples while wearing a nose-clip. EBC samples have been acquired by breathing on average for 5–10 min. The samples have been frozen to -80 °C for a period not exceeding 1 month.	They used a portable condenser (TURBODECCS; ItalChill, Parma, Italy), subjects breathed into the collecting system for 5 minutes at normal tidal volume. Samples were stored at -80 °C until analysis.
Short summary of the findings	This pilot study suggests that even short nanoTiO_2 exposure may lead to pulmonary oxidative stress; however this effect may be short-term and reversible. The clinical significance of these findings is unclear and more studies are needed.	The difference in biomarkers' concentration levels found between the two groups was statistically significant. A significant increase in the 8-OHdG biomarker present in EBC was found in patients previously exposed to carcinogenic minerals asbestos and silica. Therefore, 8-OHdG in EBC is likely to be associated with carcinogenic processes in the lungs; however, this hypothesis requires a profound evaluation on a larger sample of patients.	This study suggests that UFP content in EBC reflects airway inflammation and oxidative stress. Carbonyl and 8-OHdG levels in EBC were higher among COPD patients compared with healthy subjects. High UFP concentrations in serum of COPD patients support the hypothesis that increased epithelial permeability is responsible to low UFP content in EBC.
Quality sub-scores by domain			
I. Study sample quality	4	4	4
II. Study design quality	3	3	3
III. Analytical/ technical quality	2	6	5
IV. Quality of the data processing, analysis and result reporting	3	3	2
Final score *(sum of the points assigned to each of above-cited criteria, the highest score is 27 (3 points assigned to each of 9 criteria)*	12	16	14
Quality level (< or = 13 = Low; 14 to 19 = Moderate; > 20 = High)	Low	Moderate	Moderate

*studies included in the meta-analysis.

	Ref 7 *	Ref 8 *
Author, year, country	Sibel Doruk, 2011, Turkey	Daniela Pelclova, 2018, Czech Republic.
Title, reference	Oxidative status in the lungs associated with tobacco smoke exposure. Clin Chem Lab Med, 2011. 49(12): p. 2007-12	Exhaled breath condensate biomarkers reflect systemic changes in patients with chronic dioxin intoxication. Chemical Monthly, 2018: p. 1579-1586.
The objective of the study	To investigate oxidative stress in the lungs associated with tobacco smoke and to evaluate the effect of this stress with pulmonary function tests (PFTs).	(TCDD) is highly toxic but the involvement of the respiratory system has not yet been studied. TCDD in the blood was measured and biomarkers of oxidative stress and inflammation were analysed in 2016 in the EBC of the last eight male survivors from 80 workers intoxicated with TCDD during the production of herbicides from 1965 to 1968. The results were compared with their findings in 2010 to evaluate a trend.
The short description of the exposed/sick sub-population (who are included, participant rate and number of participants)	Group I: 26 current smokers (23 male, 3 female) with a history of smoking at least 7 packet/years, Group II: 21 subjects (15 male, 6 female) who did not smoke within the last year but had environmental tobacco smoke exposure of more than 3 h a day for at least 1 year in some microenvironments. Subjects with a history of asthma, atopy, chronic pulmonary or systemic diseases, gastroesophagial reflux, upper respiratory infection within the last 2 weeks and previous or concurrent use of vitamins were excluded.	8 male survivors from 80 workers intoxicated with TCDD during the production of herbicides from 1965 to 1968.
The short description of controls sub-population (who are included, participant rate and number of participants)	22 non-smokers (10 male, 12 female) without ETS. Subjects with a history of asthma, atopy, chronic pulmonary or systemic diseases, gastroesophagial reflux, upper respiratory infection within the last 2 weeks and previous or concurrent use of vitamins were excluded.	7 men (5 smokers, 2 non-smokers) with comparable lifestyle factors. The blood of three wives of the patients was analysed for TCDD and other dioxin-like contaminants to eliminate a possible local effect of the living location in the 1.5–10 km distance from the factory, from which, however, the old TCDD-contaminated herbicides residues have been removed more than a decade ago.
Method of 8-OHdG measurement (when EBC collected…)	EBCs were collected using a condenser Ecoscreen Turbo (DECCS, Jaeger, Germany). The subjects were asked to breath for a period of 15 min whilst wearing a nose-clip. The collected condensate was immediately stored at -70 °C.	EBC samples were collected using the Ecoscreen Turbo (DECCS, Jaeger, Germany). All subjects breathed tidally for about 15 min while wearing a nose clip. Time of collection was 15 min. All samples were immediately frozen and stored at -80 °C.
Short summary of the findings	Tobacco smoke exposure affected the balance between oxidative stress and antioxidant capacity of lungs. The mean levels of 8-OHdG in smokers were significantly higher than non-smokers. Preventing ETS exposure might decrease oxidative damage. Increased levels of 8-OHdG and SOD levels could be assessed as an early sign of airway damage.	Differences in the expression of the biomolecular markers in EBC as compared to controls were not associated with lung impairments and the respiratory parameters measured. Therefore, these EBC markers can be used to evaluate systemic oxidative stress and inflammation in tissues and the endovascular, atherosclerotic, neurotoxic, and metabolic effects of TCDD.
Quality sub-scores by domain		
I. Study sample quality	5	3
II. Study design quality	2	7
III. Analytical/ technical quality	4	3
IV. Quality of the data processing, analysis and result reporting	2	1
Final score *(sum of the points assigned to each of above-cited criteria, the highest score is 27 (3 points assigned to each of 9 criteria)*	13	14
Quality level (< or = 13 = Low; 14 to 19 = Moderate; > 20 = High)	Low	Moderate

*studies included in the meta-analysis.

c. Meta-analysis results

We split the six study samples into eight subgroups according to the analytical method used and according to the smoking status of participants (smokers/passive smokers or non-smokers). Five subgroups were not occupationally exposed to dust or other hazards and were analyzed using the chemical method. The three other sub-groups were also unexposed but analyzed using the immunochemical method. The coefficient of variation was between 22% and 41%. No study group was excluded on this basis. Figure 55 presents a forest plot of 8-OHdG concentrations in EBC for different subgroups, stratified by analytical method. For both analytical methods the between study variability was very high (over 99% of the total variability) and completely dominated the within-studies variability. By consequence, a meta-regression of all the study groups mostly reflected differences between studies rather than any actual effect of determinants considered. For that reason, a mixed model with study ID as a random effect appeared a more relevant analysis model. Nevertheless, due to small number of included studies quantitative statistical analysis of determinants were very limited. For instance, the effect of sex could not be assessed, as it was completely confounded with the analytical method (data not shown). Stratified analysis of the 8-OHdG measured in EBC using chemical analytical method showed no major difference between the non-smoking populations versus mixed populations or smokers (Figure 56a). In contrast, when the 8-OHdG was measured in EBC using immunochemical analytical method, the group of current smokers had a higher 8-OHdG GM or concentrations than the non-smokers and the passive smokers (Figure 56b).

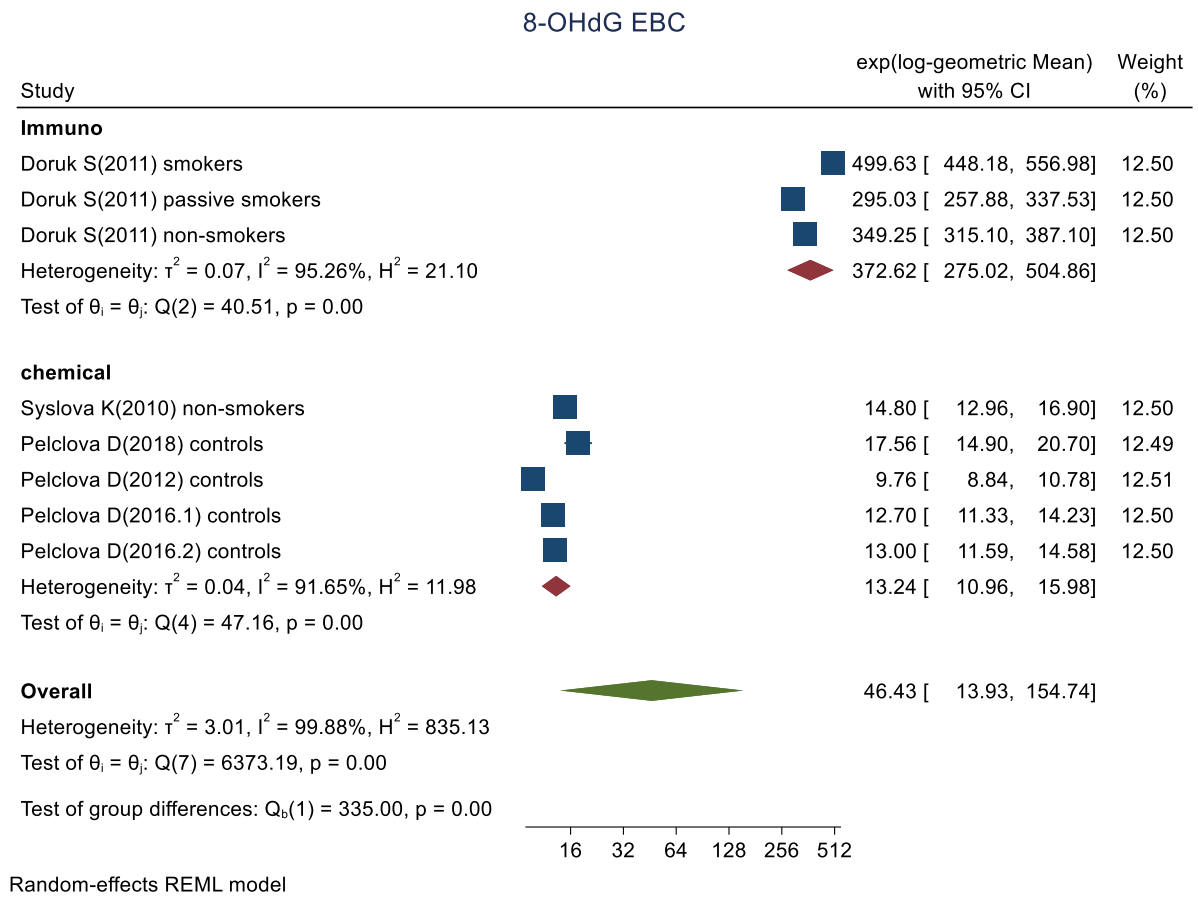


Figure 55. *Forest-plot of the 8-OHdG levels [pg/mL] measured in the exhaled breath condensate according to the analytical method*

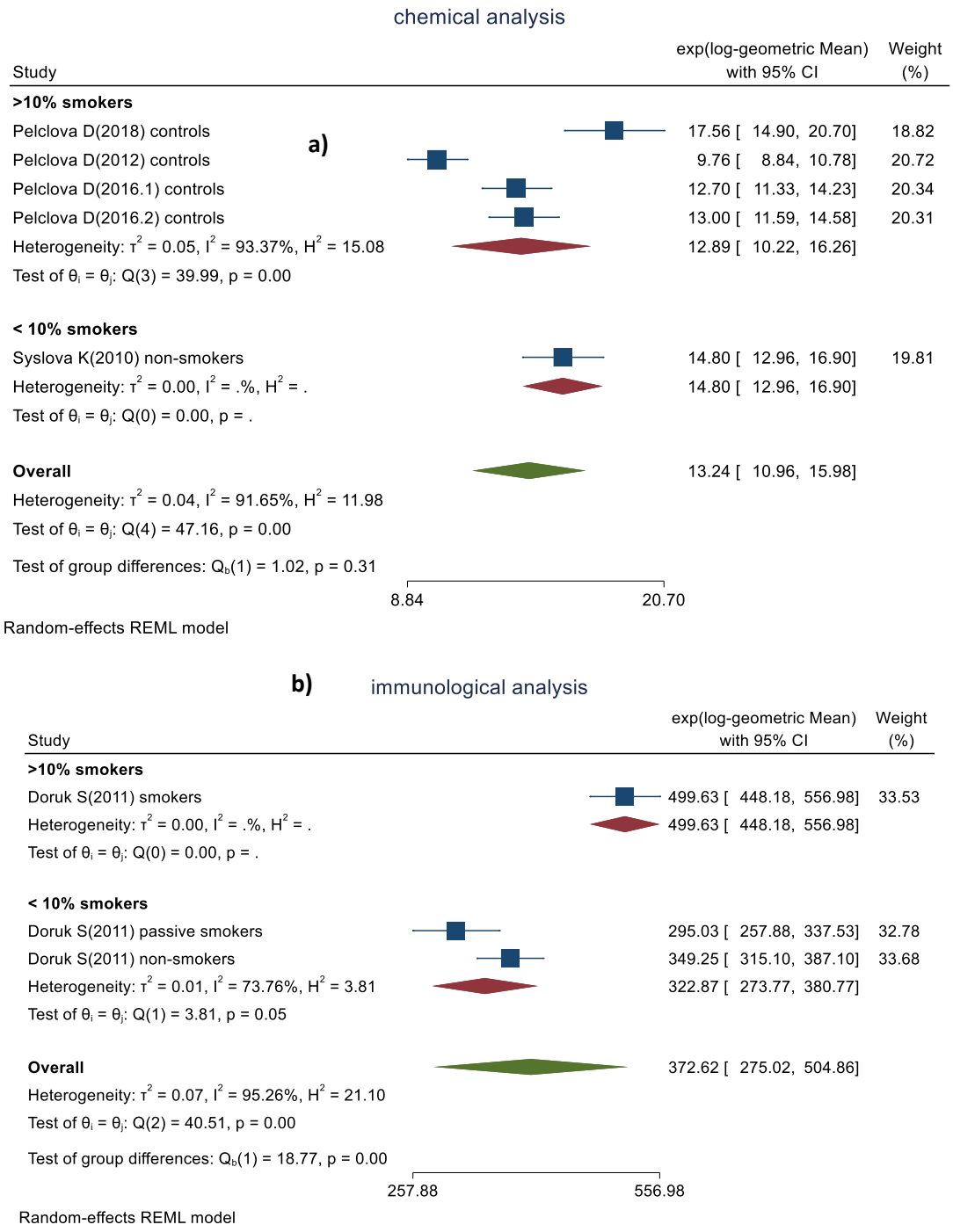


Figure 56. *Forest-plot of the 8-OHdG levels (pg/mL) measured in the exhaled breath condensate according to the smoking status and analytical method*

III. Overview results for 8-isoprostane

1. Context

In addition to 8-OHdG, another meta-analysis on 8-isoprostane was performed in EBC based on the same protocol as in the above. It was published in International Journal of Molecular Sciences: **Reference Ranges of 8-Isoprostane Concentrations in Exhaled Breath Condensate (EBC): A Systematic Review and Meta-Analysis**. Int J Mol Sci. 2020;21(11):3822. Published 2020 May 28. doi:10.3390/ijms21113822 Yara Shoman , Pascal Wild, Maud Hemmendinger, Melanie Graille, Jean-Jacques Sauvain, Nancy B Hopf 1, Irina Guseva Canu.

I will present the results in the following paragraph.
In the study on 8-isoprostane, it was notably suggested, as for 8-OHdG, that the collection method used has a significant effect on the measured concentrations of 8-isoprostane, which corroborates with our previous findings (*refer part 3*).

2. Results

a. Study Selection

We identified 19,421 studies applying the literature search string in four databases (Figure 57), of which 11,867 studies remained after removing duplicates. Restricting the search to include only EBC, gave 893 records. We excluded 548 studies based on the title and abstract screening. Finally, we retained 86 studies where 8-isoprostane was analyzed in EBC. After reading these studies thoroughly, all 86 studies were included in the qualitative synthesis including risk of bias and quality assessment [192-194,197,198,201,205,287-365], and 52 studies into the quantitative analysis [192-194,197,198,201,205,288-294,296,298-300,304,307-309,313,315-322,324,326,328-331,341,342,344,346-349,351-353,355,356,359,361,365]. In the quantitative analysis, ten studies were excluded because no information was provided to estimate variability (GSD). Another 12 studies were excluded because their coefficients of variation (CV) were beyond acceptance limits (greater than 10% and lower than 200%). Only 12 studies out of the remaining 64 reported using chemical analytical methods. We therefore decided to include only studies using immunological methods. This resulted in 52 studies for the meta-analysis.

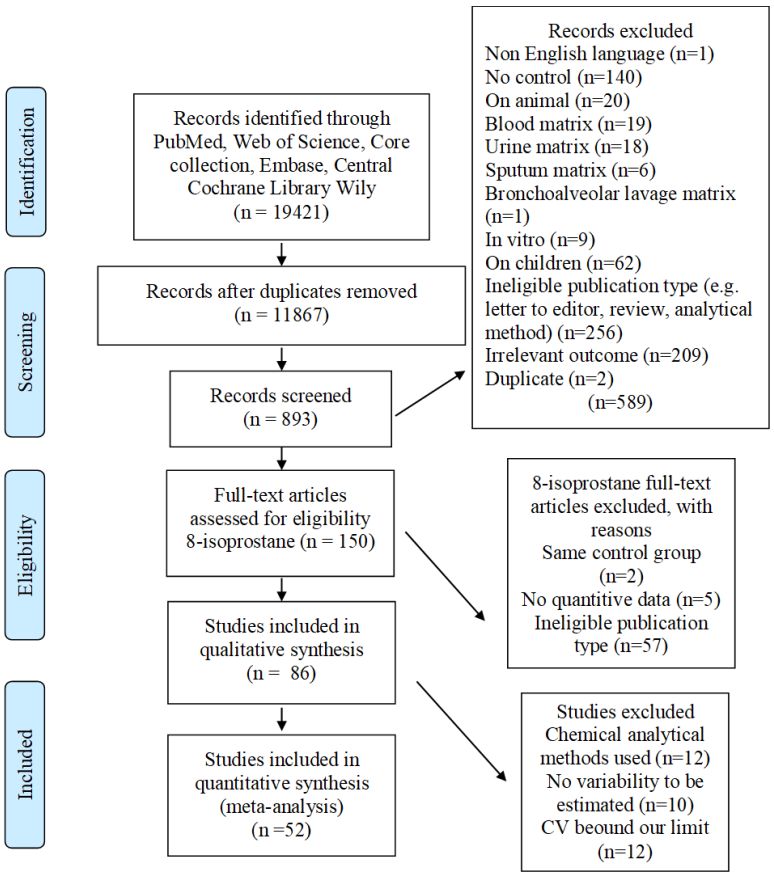


Figure 57. *Flow chart of study selection*

b. Descriptive Analysis

86 studies were included in the systematic review. These studies were conducted between 1999 and 2019 in different countries, namely USA, UK, Japan, China, Poland, Czech Republic, Finland, France, Belgium, Sweden, Taiwan, Italy, Turkey, Egypt, Israel, Iceland, and Chile. The included studies had more male participants (59%) than females (41%) and the participants had mean age 46.84 years with standard deviation 2.10. In overall, 15 studies (17.4%) were classified of low quality (high risk of bias), 57 (66.3%) of moderate quality (moderate risk of bias), and only 13 studies (15.1%) were of high quality (low risk of bias). Table 32 provide detailed information on the included high quality studies. Most of studies lacked the representative sample, they included small number of participants, and used convenience sampling. The other drawbacks were biases in the measurement of the exposure or outcome that resulted in a high risk of bias.

Table 32. *Description of the included high quality studies*

Author, Year, Country, [Ref]*	Study Objectives	Population Studied and Number of Participants	Control Population and Sub-Population	Method of EBC Collection and Analysis	Main Findings	Quality Score
Antonopoulou,2008, Greece, Ref 21*	To assess airway inflammation by measuring the levels of 8-isoprostane, interleukin-6 (IL-6), Tumor Necrosis Factor-alpha (TNF-a), and pH in EBC and study their plausible relation with plasma levels of leptin.	112 consecutive patients referred with symptoms suggestive of OSA. After a full night diagnostic polysomnography, 45 OSA patients (37 males, age 52±12 years, BMI 33.5±7, 28 smokers) finally formed the patients group. Patients with Apnea/Hypopnea Index (AHI) 10 were included in the study.	25 healthy subjects non-randomly selected, matched for age, gender, and BMI, (18 males, age 51±7 years, BMI 31±3, 15 smokers). They were mainly recruited from a population used as healthy subjects in other studies of this research group.§	EBC was collected by using a condenser (EcoScreen; Jaeger, Wurzburg, Germany). The condensate was stored at -70°C.	Increased levels of leptin were not associated with the observed airway inflammation in OSA. The observed airway inflammation seemed to be independent of smoking habit with limited association with disease severity.	20
Bastug, 2013, Turkey, Ref 24*	To measure oxidative stress in Hyperthyrod patients in EBC through measuring the levels of 8-isoprostane.	42 Hyperthyrodism patients (12 males, 30 females).	42 (12 males, 30 females, non-smokers) age and BMI matched healthy control subjects.§	EBC was collected using a condenser (EcoScreen). Subjects were asked to breath tidally for 15 min using a noseclip. Condensates were stored immediately at −70 °C.	8-isoprostane levels in EBC of hyperthyroid patients were found to be significantly higher than that in healthy control group.	20
Chow, 2009, Australia, Ref 32	To assess lung oxidative stress and inflammation in vivo in subjects with asbestos-related disorders and compare them with age matched controls.	All subjects (*n*=60) had a confirmed history of workplace asbestos exposure other than controls (*n*=26) and were classified into three groups (asbestosis, diffuse pleural thickening (DPT) and	Age and sex-matched controls (*n*=26). All control subjects were never or exsmokers without any evidence of asbestos-related or other lung disease after screening.	EBC was collected using Ecoscreen, subjects breathed tidally with nose-clip on. Condensate was collected after 10 min, the cooled condensate was	In asbestos-related disorders, markers of inflammation and oxidative stress are significantly elevated in subjects with asbestosis compared with	20

		pleural plaques (PPs). Smokers were excluded.		immediately stored at -80°C.	healthy individuals but not in pleural diseases.	
Chow, 2012, Australia, Ref 33	To investigate whether levels of Several reactive oxygen species (ROS) and Several reactive nitrogen species (RNS) in EBC of patients with PF differed significantly from age- and sex-matched controls, and whether these correlated with lung function.	20 subjects had pulmonary fibrosis (PF).	20 were normal controls (16 male, mean age±SD 55.3 ± 13.4).	EBC was collected using Ecoscreen, subjects breathed tidally with nose-clip on. Condensate was collected after 10 min, the cooled condensate was immediately stored at 80 °C.	Inflammatory and oxidative stress biomarkers are raised in patients with PF compared with controls. EBC may be useful for detecting and monitoring lung inflammation in PF.	21
Emilsson, 2016, Iceland, Ref 37*	To investigate the association between nocturnal gastroesophageal reflux (nGER) and respiratory symptoms, exacerbations of respiratory symptoms, lung function and Sleep-disordered breathing (SDB).	This study is based on a 20 years prospective, population-based cohort study in Iceland. Among the 522 subjects contacted, a total of 455 participated, or 87% of those invited. Of the 455, 82 had symptoms suggestive of nGER. These 82 subjects were invited for a second visit in 2013, of which 71 (87%) participated.	Age and gender paired controls without any nGER symptoms (participation rate 78%, *n*=42, Female 48%, mean age±SD 56.4 ± 7.0).§	EBC samples were collected with ECoScreen II. Participants wore a nose-clip and used tidal breathing for 15 min. The samples were immediately frozen at −20 °C, and within four hours moved to −80 °C for storage.	In a general population sample, nGER is associated with symptoms of asthma and bronchitis, as well as exacerbations of respiratory symptoms. In addition, nGER is associated with increased respiratory effort during sleep.	21
Hoffmeyer, 2012, Germany, Ref 44	To evaluate subclinical changes in otherwise healthy current welders with the majority practicing this profession for decades.	58 welders (all male, 27 smokers) from the cross-sectional study WELDOX were examined. Welders were processing mild steel applying gas metal arc welding with solid wire (GMAW) or flux cored wire (FCAW).	NA	EBC was collected after shift with the commercially available temperature-controlled device ECoScreen2. The collection time was exactly 10 min.	An enhanced irritative effect in the lower airways of mild steel welders due to the application of FCAW compared to GMAW, most likely associated with a higher emission of welding fumes.	21

Inonu, 2012,Turkey, Ref 45*	To evaluate the differences in the burden of oxidative stress in patients with COPD, smokers, and non-smokers by measuring H2O2, MDA, and 8-isoprostane levels in the EBC samples.	The subjects in Group I (*n*=25) had COPD (all ex-smokers).	Group II (*n*=26) were healthy smokers (mean age±SD 61.2 ±6 y, all males) and Group III (*n*=29) were healthy nonsmokers (mean age±SD 60 ± 8 y, all males).§	EBCs were collected using a condenser (EcoScreen). The subjects were asked to breathe while wearing a nose clip, for a period of 15 min. The samples were immediately stored at 70 °C. All EBC samples were collected between 2 PM to 4 PM.	Even if respiratory function tests are within normal limits, oxidant burden in lungs of smokers is equivalent to that in COPD patients. 8-isoprostane could be useful in assessing symptom severity and health status of COPD patients.	23
Lehtimaki, 2010, Finland, Ref 53*	To find out if borderline parenchymal changes on HRCT in subjects with moderate to heavy asbestos exposure are related to the degree of pulmonary inflammation.	Of the 104 asbestos-exposed men recruited,33 were excluded based on the exclusion criteria. 35 subjects had normal parenchymal findings on HRCT and 31 subjects had borderline parenchymal changes.	41 healthy men (mean age 63) not exposed to asbestos or other harmful agents.§	EBC was collected during 15 min of tidal breathing with Ecoscreen condenser while wearing noseclips. The samples were stored at -70°C.	Borderline parenchymal changes on HRCT in asbestos-exposed subjects are associated with increased markers of pulmonary inflammation. Such borderline parenchymal changes are likely a mild or early form of the same pathological process that leads to asbestosis.	20
Pelclova, 2007, Czech Republic, Ref 65	To measure 8-isoprostane, leukotrienes B4, C4, D4, and E4 in exhaled breath condensate in patients with silicosis.	Patients with silicosis (*n*=60, 58 men and 2 women).	The control group was composed of 25 subjects (23 men and 2 women), previously working as office employees and safety inspectors, never occupationally exposed to fibrogenic dusts.	EBC samples were collected using the EcoScreen. Each subject was asked to breathe through the collection kit for 15 min with more than 2 mL of EBC collected. Samples were immediately frozen after collection (-80°C)	No significant effect of smoking or alcohol consumption on the markers examined was seen. This is the first study using exhaled breath condensate analysis in patients with silicosis.	20

Pelclova, 2008, Czech Republic, Ref 64	To investigate the hypothesis that oxidative stress due to asbestos is the main cause of increased 8-isoprostane in EBC.	92 asbestos-exposed subjects were examined (46 women and 46 men).	The control group was represented by 46 subjects (23 men and 23 women), employed as hospital technical workers (gatekeepers, adjuncts and helpers, hospital mailmen, etc.) without occupational exposure.	EBC samples were collected using the EcoScreen. Each subject was asked to breathe through the collection kit for 15 min with more than 2 mL of EBC collected. Samples were immediately frozen after collection (-80°C)	Measurement of 8-isoprostane in EBC is a promising non-invasive means for assessing the activity of asbestos-induced diseases.	20
Sood, 2013, USA, Ref 89*	To evaluate EBC 8-isoprostane concentrations following allergen-induced bronchoprovocation in asthma.	Eight mild atopic asthmatics (5 women)	Six healthy controls (four women): the majority of enrolled subjects were premenopausal overweight women (age mean±SD 39.9±9.7)§	EBC was collected using an R-tube and condensate was collected during a period of 20–30 min. EBC was stored at −70°C.	EBC 8-isoprostane concentrations do not acutely change following bronchoprovocation in subjects with mild asthma.	20
Vizcaya, 2013, Spain, Ref 95	To evaluate associations of domestic and occupational use of cleaning products with asthma and biomarkers of respiratory health.	42 cleaners with a history of asthma and/or recent respiratory symptoms (participation rate 60%).	53 symptom-free controls (participation rate 44%)	EBC was collected using an EcoScreen condenser. Collection was performed from 09:00 to 10:00 in the morning. Each subject was asked to breathe into the device for 10 min while wearing a nose clip. The samples were stored at –70°C.	Asthma in cleaning workers is characterized by non-reversible lung function decrement and increased total IgE.	21
Zhao, 2008, Japan, Ref 98*	the relationship between the pH of EBC and the concentration in EBC of a marker of oxidative stress,	Adults aged 18 years or over with asthma were	Sex-matched and age-matched healthy volunteers without respiratory disease were	EBC was collected using an EcoScreen condenser. Collection was	Stress and oxidative stress assessed by pH and 8-isoprostane concentration, respectively, in EBC did not	20

	8-isoprostane, was investigated. The relationship between these markers and lung function was also studied.	recruited (*n*=44, 20 females, nonsmokers)§	recruited as control subjects (*n*=20, 8 females, nonsmokers).	performed from 09:00 to 10:00 in the morning. Each subject was asked to breathe into the device for 10 min while wearing a nose clip. The samples were stored at –70 °C.	show parallel changes associated with asthma and were not correlated with lung function in asthma patients.	

** Studies included in the meta-analysis; § Subgroups included in the meta-analysis; Note. IL-6 = interleukin-6, TNF-a = Tumor Necrosis Factor-alpha, LTB4 = Leukotriene B4, DPT = diffuse pleural thickening, PPs = pleural plaques, OSA = Obstructive sleep apnea, ROS = reactive oxygen species, RNS = Several reactive nitrogen species, PF = pulmonary fibrosis, SDB = Sleep-disordered breathing, nGER = nocturnal gastroesophageal reflux, OSAS = sleep apnea–hypopnea syndrome, GMAW = gas metal arc welding with solid wire, FCAW = flux cored wire, HRCT = high-resolution computed tomography, and IgE = immunoglobulin E.*

c. Meta-Analysis

We conducted the meta-analysis on healthy subjects aged 18+ years, exclusively. Participants were divided into subgroups depending on their gender. In total, within the 52 studies included in the meta-analysis, we considered 62 subgroups of participants included, with 1980 participants in overall. We had 322 participants in the males only subgroup, and 12 participants in the females only subgroup. The mean age of the subgroups was 44.90 years with standard deviation (SD) 11.61. Participants were 54% males and 46% females. Only 18 studies reported BMI [192,193,288,289,293,296,300,307,312,319,320,324,331,349,351,353,356,361]. Among them 65% reported BMI higher than 25. The between-study heterogeneity was very high (I^2 = 99.22%) and the mean 8-isoprostane concentration was 7.97 pg/mL with a 95%-confidence interval (95% CI) between 6.46 and 9.85. The heterogeneity remained high even after stratifying by gender, I^2 = 99.19% for studies with mixed gender groups (both males and females) (Figure 58) and I^2 = 99.20% for studies with specific gender groups (only males or females) (Figure 59). Due to the high heterogeneity, a meta-regression of all the study groups mostly reflected differences between studies rather than any actual effect of determinants considered. For that reason, a mixed model with study ID as a random effect appeared the most relevant.

Our results showed no significant difference in the 8-isoprostane concentrations in EBC of healthy subjects with respect to gender, age, BMI, or smoking status (Table 33). However, there was a significant difference related to the device used in EBC collection. Measured concentrations were higher when EcoScreen device was used for EBC collection: GM (95%CI): 7.67 pg/mL (5.58–9.76) compared to Rtube device 3.42 pg/mL (0.57–6.27). In nine studies, the name of the device used was not reported [194,201,290,291,294,312,347,348], one study used TurboDECCS [309], and two studies used home-made device [328,329]. In these studies, the concentrations of 8-isoprostane was 14.01 pg/mL (7.03–20.99). It is worth to mention, that after adjusting this model for other factors characterizing the EBC collection and storage, the effect of device became statistically non-significant. None of adjustment factors had significant effect per se (Table 34), which raises question of their importance in frame of standardization guidelines. Table 35 shows 8-isoprostane concentrations when measured using different devices and stratified by gender but the results showed no significant difference.

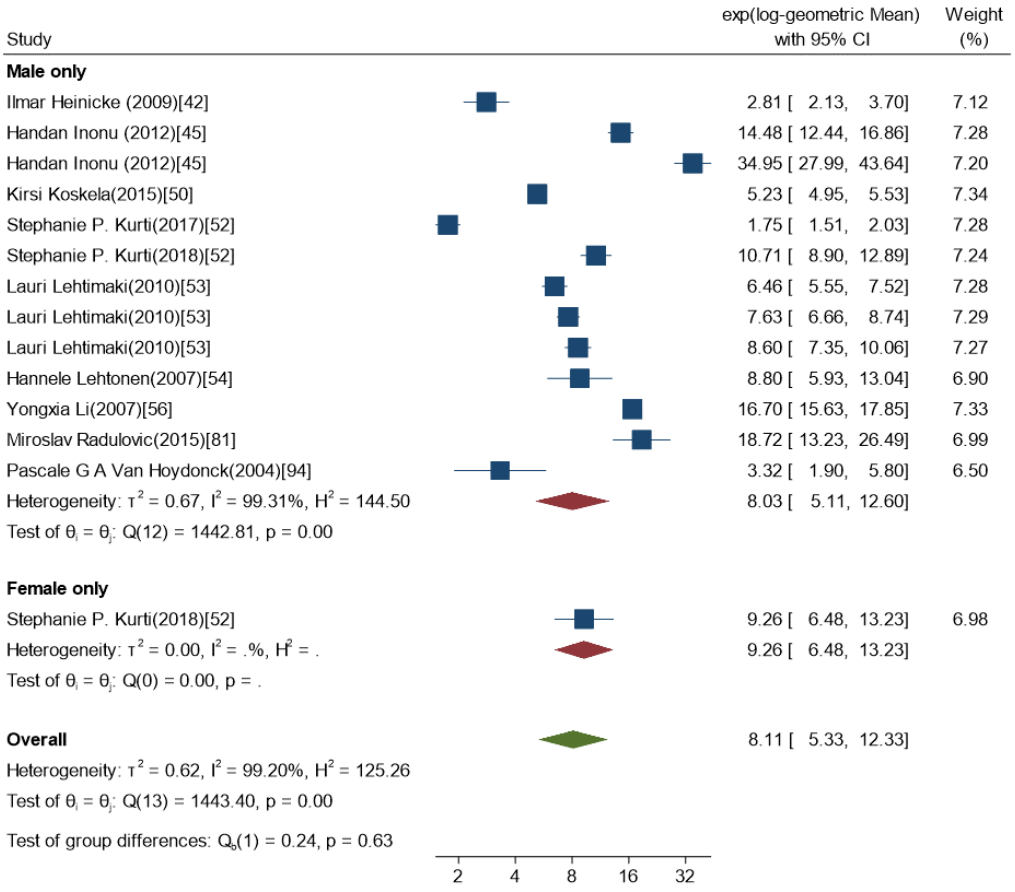


Figure 58. *Forest-plot of the 8-isoprostane levels [pg/mL] measured in the exhaled breath condensate using immunological analytical methods and in studied with only females or only males (n=13)*

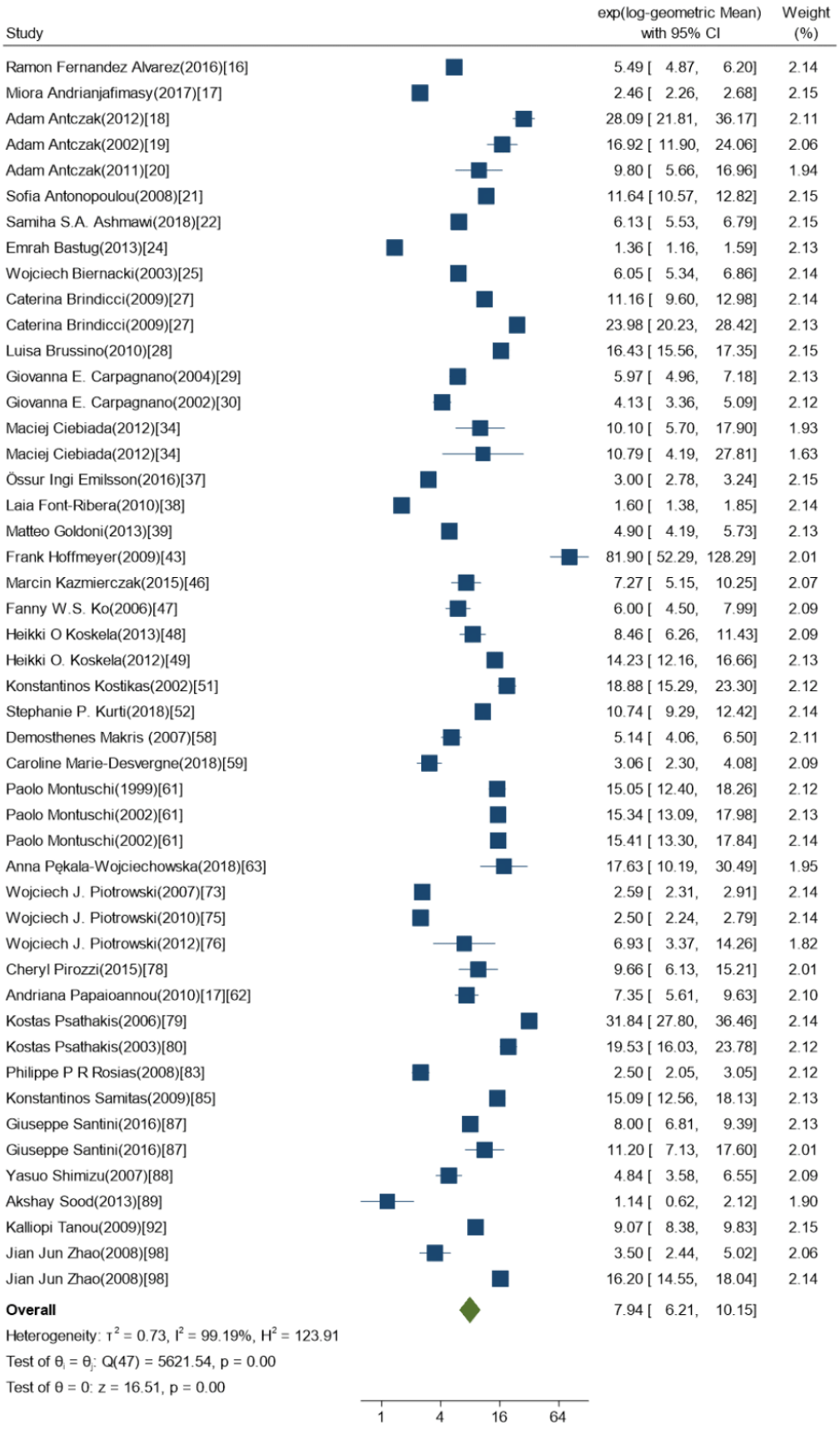


Figure 59. *Forest-plot of the 8-isoprostane levels [pg/mL] measured in the exhaled breath condensate using immunological analytical methods and in studies with both males and females (n=39)*

Table 33. *Mixed effect regression analysis to investigate the relation between levels of 8-isoprostane in EBC [pg/mL] and device used during collection, gender, BMI, smoking status, and mean age of the population*

LogGM	Regression coefficient	P>|z|	95%CI
Device			
Ecoscreen	0		
NA/others	0.64	0.03	0.06–1.21
Rtube	−0.70	0.13	−1.60–0.21
BMI			
Gender			
Males only	0		
Males and females	−0.39	0.38	
BMI<25	0		
BMI>25	−0.19	0.71	−1.25–0.85
No BMI reported	−0.08	0.87	−0.88–1.04
Smoking			
Non-smokers	0		
Smokers and nonsmokers	−0.24	0.43	−0.86–0.37
Smokers	0.10	0.76	−0.56–0.76
Mean age			
<40	0		
40–60	0.02	0.94	−0.48–0.52
>60	0.02	0.96	−0.82–0.87
Intercept	2.31	0.00	1.26–3.36
Between-study standard deviation	0.56		0.25–1.17
Within-study, between-group standard deviation”	0.55		0.29–1.02

*Seven studies comprising nine subgroups are excluded from this analysis because of missing values. * p<0.05 corresponds to chi-square test*.

Table 34. *Mixed effect regression analysis to investigate the relation between levels of 8-isoprostane in EBC [pg/mL] and device used during collection, BMI, smoking status, and mean age of the population*

LOGGM	Regression Coefficient	P>|z|	95%CI
Device			
Ecoscreen	0		
NA/Other	0.47	0.113	0.11–1.05
Rtube	0.69	0.098	1.5–0.12
Temperature			
−20	−1.31	0.219	−3.40–0.78
−60	−0.12	0.984	−1.26–1.24
−70	0		
−80	−0.14	0.565	−0.66–0.36
NA	−2.73	0.007	−4.73-(-0.73)
Nose-clip			
No	0		
Yes	−0.55	0.119	−1.250.14
Duration			
10	0		
10–15	0.35	0.539	−0.77–1.48
15	−0.07	0.767	−0.55–0.40
20	0.36	0.550	−0.81–1.52
Time			
Afternoon	−0.47	0.431	−1.64–0.70
NA	−0.44	0.12	0.1–0.12
Morning	0		

Table 35. *8-isoprostane reference ranges [pg/mL] in the exhaled breath condensate (EBC) of healthy adults**

EBC Device	Males Only	Males and Females	All Population
Rtube	6.23(1.75–10.71), (*n*=2)	6.36(2.46–10.74), (*n*=6)	9.26(2.46–10.71), (*n*=9)
Ecoscreen	18.7(8.6–23.0), (*n*=17)	8.00(4.13–14.23), (*n*=35)	9.44(5.73–19.15), (*n*=52)
NA/Other	2.81(2.81–2.81), (*n*=1)	15.41(6.12–19.52), (*n*=11)	15.37(5.63–19.20), (*n*=12)

** Results are presented as median (Interquartile range (IQR)), number of subgroups (n), only one subgroup had females only and the median was 9.26 pg/mL but no variability can be estimated.*

GENERAL DISCUSSION

I. Methodological aspects

Refer to part 2 for details of the results

For epidemiological purpose, we optimized and validated sensitive and accurate methods to quantify MDA, 8-OHdG and 8-isoprostane concentrations in EBC samples.

1. 8-OHdG/8-isoprotane method optimization

The development was based on [366,367], but we optimized it to simultaneously quantify both biomarkers for a fast and cost-effective analysis. We tried to purify the EBC prior to analysis because this matrix contains a large amount of proteins (in a typical range of 0.76-107.7 µg/mL) [245] that might suppress the quantification of the two biomarkers. Nevertheless, this procedure was abandoned, as both biomarkers have a too large difference in polarity – *8-OHdG* has polar functional groups (amides, hydroxyls, and amine), in contrast to 8-isoprostane, mostly formed by apolar alkane chains - which would have reduced further the amount of compounds already present at low concentrations [368].To achieve the required sensitivity we decided to concentrate as much as possible our sample and considered two viable approaches: lyophilization or centrifugation in conjunction with low binding plastics or glass. The concentrations obtained using lyophilization were much lower (20-40%) compared to the centrifugation approach while the evaporation times were similar. This demonstrated a possible evaporation of the biomarkers at very low pressures. Consequently, we chose to use centrifugation approach and low binding plastic materials when concentrating EBC samples.
We demonstrated satisfactory method performances: LOD < 1 pg/ml for both biomarkers, excellent recoveries for 8-isoprostane (95–104%) and good for 8-OHdG (89-98%) as well as adequate repeatability (< 20% for lowest concentrations). The matrix effect was negligible when comparing slopes from calibration curves prepared in EBC and the standard sample. (8-OHdG p=0.20; 8-isoprostane p=0.83) demonstrating an absence of potential interferences in the analysis, as expected for EBC [369].

2. MDA method optimization

We experienced interference concerns during the development of the method impacting its sensitivity. This interference was identified as unwanted MDA-DNPH signal, present in the procedural blank. It was quite variable between the different experiments even though we

worked under strictly comparable conditions. We identified the DNPH reactive solution at the origin of this interference signal. We hypothesized that compounds such as acrolein [370], ozone [371,372] nitrogen dioxide [373] or acetone [374] naturally present in the air [375] would interact with its environment (e.g., water droplets) to form ambient MDA and would come into contact with our solvents or the DNPH reagent. This latter phenomenon is supported by the fact that DNPH is moistened with 4% water. However, no ultrapure DNPH exists since it naturally reacts as a "passive cartridge" with MDA present in the air. We have made several attempts to remove the contamination of the procedural blank (e.g., liquid-liquid extraction, solid phase extraction, crystallization) but with no success. The use of separation chromatography to purify DNPH solution was not investigated due to time constraints. Nevertheless, some conclusions have emerged from these experiments. To minimize interference, we recommend to use as little organic solution as possible, use acetic acid instead of strong acids and use a daily fresh DNPH solution. We also suggest to consistently report contamination level in the situation where a significant procedural blank is found in samples, otherwise it would difficult to compare the results between studies, as some might be overestimated. So far, very few publications [376] mention the presence of MDA-DNPH interference signal in the procedural blank samples.

To assess the performance of the optimized method, we thoroughly took into account the contamination in our calculations because it had a significant impact on the samples. Firstly, to calculate our calibration curves we applied a $1/x^2$ weighting, which provided much more importance to the values obtained at low MDA concentrations that are subject to so much more fluctuation due to contamination. Then, to determine LOD, as the contamination producing a variable signal, we calculated LOD based on three times the error at the origin divided by the slope of the calibration curve. This approach may appear as very conservative compared to other LOD calculations (Table 36), but it ensured to accurately quantify MDA even when the contamination level was extreme. There are several other methods for calculating the LOD, including (i) the average noise plus three times the standard deviation of the noise signal in independent measures of a blank; (ii) three times the standard deviation of repeated independent measures in a low concentration standard at the lowest point in a calibration curve [377]. Finally, the contamination was quantified and then subtracted from the final values found in all samples. This step seldom described in the scientific literature is yet critical because depending on whether the blank is subtracted or not, the values can greatly vary, which can make it difficult to compare results between studies or even mask a slightest inter-individual variation in MDA concentration, which is often the case in exposure studies on healthy people.

Table 36. *Value of LOD resulting of three common methods of calculation*

(i)	(ii)	**Current LOD calculation**
31 pg/ml	39 pg/ml	**71 pg/ml**

(i) = LOD method using the average noise plus three times the standard deviation of the noise signal in independent measures of a blank; (ii) = LOD method using three times the standard deviation of repeated independent measures in a low concentration standard at the lowest point in a calibration curve.

In this respect, we calculated the uncertainty to examine whether this contamination could mask inter-individual variation in our EBC samples. In the present study, almost 30% of MDA concentrations in the volunteers of this study were <LOQ. At this level, the contribution of contamination can be critical on the values.
Nevertheless, we found the analytical variability determined at 23% remained far lower than the inter-subject of 41-57%. This confirms that it will be possible to detect relevant inter-subject differences in future epidemiological studies. To our knowledge, only Corradi et al., [209] reported a value attributed to inter-individual variability of MDA in EBC. This value found at 18.5%, was lower than the one we determined in our study. For other matrices such as urine, Martinez et al., [378] reported higher variabilities for MDA, with inter-individual variabilities above 300%.

Concerning intra-individual variability, we observed that about 38% of the observed intra-individual could be attributed to the calculated analytical uncertainty. This variability would have a significant impact in studies relying on within-subject differences. This effect is even greater when the samples are not analyzed with the same preparation. This clearly indicates that the quantification of MDA levels in EBC is still quite challenging in term in sensitivity, and requires to develop method to remove the interference signal before using it in longitudinal studies where samples are analyzed sequentially.

3. OPEA optimization

The optimization of the OPEA determination approach has significantly improved its robustness and repeatability of the measurements. A major issue we encountered was the instability of the blank value with H_2O that required several sets of experiments in order to be identified and solved. In a first stage, the formation of microbubbles (not visible by eye) in the reaction vial during vortex was generating strong optical interferences that were particularly

hard to discriminate from the expected absorbance change. The introduction of an internal reference with the near-IR light source dramatically reduced such interferences. Another issue was directly related to the one-shot configuration of the OPEA assay where the exhaled air sample is directly bubbled into the reaction vial that implied the use of needles/septum system. In such configuration the introduction of air sample into 1 mL vial (at the flow rate of 250 mL/min) produced considerable amount of splashes that disturb the stability of the measurements. Finally, deuterium oxide was found to be particularly suitable since the corresponding blanks are close to the "zero" value and show high reproducibility. The two-steps approach enables achieving good reproducibility of the measurements and performing triplicate per sample. The compromise between ease-of-use and analytical robustness has been challenged in this part of the work.

Another issue concerned the analytical treatment of the raw data generated by the photo-sensor that until recently required a considerable amount of time. In the frame of this thesis, a new data treatment tool – so called Splunk® - has been implemented in our laboratory, allowing us to automatically process information in real time and in a very easy way. In addition, the software enables the visualization of the slopes corresponding to each measurement file in order to identify any artefact. So far Splunk represents a clear advantage to generate faster high quality data analysis. However due to license policy (not open source) that conflicts our aim to guarantee users completely open access to the device, we are still working on developing our own data extraction solution for the future.

II. Experience gained from the method application on the pilot field study

1. 8-OHdG/8-isoprostane

We failed to detect our biomarkers when the method was applied to EBC samples in pilot field study, despite the fact that the analytical method itself was sensitive enough. Many factors can be responsible for the lack of results. These factors are considered here under.

a. Storage and collection time

Storage conditions can influence the analyte levels [379]. In this field study, EBC samples could not be rapidly analyzed as the place of collection and analysis were distant (Paris-Lausanne). To prevent artifactual changes in concentrations of biological markers after

sampling, the EBC samples were rapidly kept at -20 °C on field (Porte de Villette) and then transferred to -80 °C at the end of each collection day at RATP's Service Santé Travail et Prévention (SSTP). Storage temperature of -80 °C is considered to provide the best storage temperature [220] but after a period of time elapsed after freezing - eight months for this study, these biomarkers can still be subject to variable and poorly controlled variation [379]. Nevertheless, if we assume the same decay rate as reported by Syslova, our results would have only underestimated the concentration of 8-isoprostane up to 14% depending on the amount of EBC obtained from the workers. For another author [380], 8-isoprostane can even be analyzed 5-9 years after collection. 8-OHdG is reported to be stable in water for several months at 4 °C [381] and at least two years when stored in acidic conditions such as in urine at -80 °C [382]. Given those results, we cannot conclude that storage time is responsible for the large number of “not detected” results we have obtained.

The EBC collection device used in this study has been suggested as a factor hampering quantification of biomarkers such as 8-isoprostane [239,241,269,366] (see part 4). Potential loss of biomarker can occur due to adsorption on the polypropylene inner surface of the TurboDECCS collection tube [383]. Some authors performed studies to check whether there was a difference between glass and plastic-based collection device [383] or tried to passivate the surface of the condenser with different compounds (Tween-20, bovine serum albumin) [241,384]. However, the latter results were inconsistent. On our side, (see part 2) we could not confirm that the undetected levels of 8-isoprostane and 8-OHdG in our EBC samples were due to adsorption of the analytes on the surface of the used material.

b. Protein interferences

The proteins present in EBC are concentrated during the preparation of the samples and might suppress the quantification of both biomarkers. Protein extraction could be necessary prior to sample analysis. However, in the Gonzalez-Reche et al.’s study [385] the exclusion of proteins (≥ 17 kDa) did not significantly improve the quantification of 8-isoprostane in EBC obtained from healthy volunteers. We are not sure that reducing protein contamination could even really improve the LC-MS signal.

The proteins present in EBC could also conjugate with 8-isoprostane and make it undetectable [386]. Specific enzymes could be used to release the biomarker, but the protocol would involve drastically diluting the EBC, which is hardly practicable. it is worth noting that the problem of conjugation has never been reported in the scientific literature except in plasma, where 8-isoprostane is bound to plasma lipids [387] and in urine, where 8-isoprostane is glucuronide

conjugated and the amount conjugated vary between 30 and 80% of the total 8-isoprostane levels.

c. Variability in dilution of respiratory droplets

The measurement of biomarkers in EBC stems from the idea that the biomarkers present in airway lining fluid droplets would become aerosolized during exhalation [181]. These two oxidative biomarkers are poorly volatile, which implies that a major part could remain associated with surfactant in the lungs [186,189]. This could be an explanation for their absence of detection in the condensate.

d. Is 8-OHdG really present in EBC?

Regarding 8-OHdG, its presence in EBC is still debatable [388,389], since a very limited number of studies have reported concentrations in this matrix and the results are very inconsistent (see part 4). Only one team managed to quantify 8-OHdG by LC-MS/MS (Syslova and Pelclova being part of the same laboratory), which is considered as the gold standard method. Two others teams have analyzed it by immunoassays. The fact that we failed to find 8-OHdG in any of our samples suggests that this biomarker would be present at very low concentrations in the lung. Another hypothesis would suggest that 8-OHdG produced at the pulmonary site may be excreted from the lung and directly circulate in the bloodstream. This is in contradiction with data from the Syslova group [237] who reports concentrations between 12-19 pg/mL. Another hypothesis is that 8-OHdG would be rapidly adducted with lipids such as malondialdehyde present in the airway lining fluid and would therefore be impossible to detect without prior separation [158].

Finally, a lot of work is still needed to be done before 8-OHdG/ 8-isoprostane can be accepted as good biomarkers of oxidative stress and this technique can be considered for applications in the clinical setting. Our two meta-analyses on 8-OHdg and 8-isoprostane (*refer part 5*) have failed to provide baseline values for these both BM due to an excessive heterogeneity in the values of these BMs reported in the literature.

2. MDA

We were able to detect MDA in all the EBC samples, but at very low concentrations close to the LOQ. Overall, 73.6% of the values were above the determined LOQ of 211 pg/ml and 25.6% having concentrations between LOD and LOQ 1.2% were below the LOD. The values of MDA concentration in EBC obtained by derivatization with DNPH, ranged between < LOD to 886.2 pg/mL and were lower than those reported in the literature using the same chemical derivatization conditions *(*Table 37*).*

Table 37. *Descriptions of the analytical methods previously developed to analyze MDA in EBC with DNPH derivatization*

Reference	Technique	EBC volume [µl]	LOD [pg/mL]	LOQ [pg/mL]	n[a]	EBC levels [pg/mL]
This study	LC-ESI-MS/MS	125	70	211	164	115 (<LOD-886)[b]
[376]	LC-ESI-MS	100	42	126	205	491 (294-711)[c]
[373]	LC-APCI-MS/MS	100	72	216	12	806 (77-1685)[b]

[a] : number of samples analyzed
[b] : median with the range in parentheses ;
[c] : median above LOD with interquartile range into parentheses

Such difference could be due to the absence of blank correction or different collection systems and procedures that would generate differently diluted breath condensates [220].

Relevance of MDA as biomarkers of effect

We did not observe a clear effect of the work-shift on the MDA concentrations in EBC, indicative of a rather constant physiological oxidative activity and an absence of a preferred time to collect the biomarker. This latter finding remains to be confirmed as it is possible that the peak of excretion was at noon and that we missed it (we only had two measurements per day). An experimental laboratory study should be conducted to examine the kinetics of this BM and its excretion in urine and EBC over 24 hours.

The type of occupational activity nevertheless had a significant impact on the level of malondialdehyde in EBC. The station agents exhibited the highest MDA concentrations, suggesting greater of oxidative stress in their airways. Interestingly, the station agents had also the highest particle number concentration exposure (*refer DiSCmini values in part 4*). Our finding is in line with in vitro studies showing that transition metals enriched PM, similar to

subway PM [390] can induce oxidative stress and inflammation. The increase of MDA was associated with elevated levels of acetate and a higher ratio NO_2^-/NO_3^-. Indeed, a metabolic adaptation may arise in order to face oxidative stress. This antioxidative cellular defense linking metabolism to mitigate oxidative stress has been recognized only recently for bacteria [391].

Acetate is a short chain fatty acid. These molecules are known to regulate inflammation by acting on the recruitment of circulating leukocytes to the inflammatory site via adhesion on endothelial cells [392] and to maintain the intracellular redox balance [393]. In the presence of ROS and altered redox metabolism, the cell induce the production of acetate as compensatory pathway to maintain its energy requirements through the oxidative decarboxylation of pyruvate [393]. This acetate is then used in replacement in the mitochondria, allowing the constant formation of acetyl-CoA, major metabolic source of cell energy [394,395]. Acetate is also involved in the inhibition of maturation of stress granules (membrane-free organelles formed by of translationally blocked mRNA that appear when cells are subjected to environmental stresses) implicated in pathogenesis of inflammatory diseases [396,397]. The positive but rather weak correlation between acetate and MDA could be an argument in favor of this oxidative decarboxylation of pyruvate in presence of ROS. An increase of acetate levels in EBC was reported for different diseases presenting an important inflammatory component like chronic obstructive pulmonary disease [394,398].

MDA was also associated higher ratio NO_2^-/NO_3^-. Nitrite (NO_2^-) and nitrate (NO_3^-) are considered as a storage pool for NO production and other bioactive nitrogen oxides [399]. During metabolic stress, when the oxygen-dependent NO synthase enzyme might be compromised, NO production is ensured by reduction of NO_2^- through involvement of haemoglobin [400], myoglobin [401], ascorbate [402] or xanthine oxidoreductase [403] among others. The increased NO_2^-/NO_3^- ratio suggests the oxidized form (NO_3^-) is less favored than NO_2^-. This increased ratio corresponds to an attempt of the lungs to compensate the locally hypoxic state (reaction of NO with deoxygenated haemoglobin, vasodilatation), systemic inflammation induced by oxidative stress. NO_3^- can also independently reduce ROS generated by mitochondria to limit cellular injury during oxidative stress [399].

Concentrations of MDA in EBC varied depending on the PM_{10} fraction and time after exposure. At lag 1, we observed that the PM_{10} mass was associated with an increase level of MDA, lactate during the shift. These results suggest a build-up of MDA during the night, in response to the PM_{10} exposure of the previous day. Differences in “timing” of action PM_{10} could be attributed to their different fate when deposited in the lungs. After inhalation, the particles will be internalized by phagocytosis within the alveolar macrophages. We hypothesized that these particles will gradually solubilize in the phagolysosome under the influence of an acid pH. A

small fraction will then be released from the phagosome and exert its toxic action inside the cell in a time-dependent reaction [404,405].

In conclusion, for this moment, among the three biomarkers assessed in this project, only MDA seems to be recommendable for measuring oxidative stress in EBC provided that high-sensibility equipment is used, and the EBC sample collection, transport, storage and pre-analytical treatment are well standardized in accordance with current recommendations (*refer part 1, V, 2. d.§ Standardization of EBC sampling and storage)*. The last condition is crucial, since the currently available studies often fail to respect the recommendation and lack standardization, limiting the possibility to derive the reference value distribution and reference interval (*refer part 5- MDA results*). Measurement of 8-OHdG and 8-isoprostane in the field is not possible yet. We therefore recommend conducting inter-laboratory studies to standardize the chemical analytical methods as well as EBC collection devices for analysis of 8-OHdG and 8-isoprostane and kinetic studies of these molecules and their elimination in EBC, measurable over 24 hours.

3. OPEA

The main optical improvement made the approach particularly suitable for the analysis of highly diluted samples such as exhaled air. The OPEA analyzer was implemented in a pilot field study conducted in Paris Subway to test if valuable biochemical information related to respiratory oxidative stress could be correlated to trends of exposures to different PM fractions. Results could not find any correlation between OPEA values and other biological or exposure variables except for Barium. This negative association with Barium ($p=0.028$) is difficult to explain from a biological point of view. Barium in subway environment is mainly in the form of sulphate and carbonate derived from manufacturing processes using barite in train brake pads [406]. Barium carbonate is toxic to humans because of its effect on gastrointestinal tract, resulting in abdominal cramps, nausea, and vomiting, following by hypokaliema. These effects may result in ventricular tachycardia, hyper/hypotension and muscle weakness/paralysis. In contrast, Barium sulphate is nontoxic to humans by ingestion (too insoluble to penetrate the gastrointestinal barrier). Following inhalation exposure, Barium carbonate salts can cause bone deposits with osteosclerosis mainly in the maxilla and femur [407]. Barium sulphate cause pneumoconiosis, bariteosis following its prolonged accumulation in the lungs [407]. These effects should theoretically increase the oxidative potential locally.

This general insensitiveness to the exposure or its negative correlation might highlight the efficacy of acute adaptive lung mechanisms regulating oxidative stress homeostasis.

The enhanced occurrence of ROS does not inevitably drive the organism into a state of acute oxidative stress. There are several effective acute adaptive lung mechanisms regulating oxidative stress homeostasis (see introduction). The formation of ROS could be neutralized by the endogenous pool of antioxidants. In such case, the presence of free radicals would only lead to lung inflammation at an early stage of the pulmonary disease. A recent meta-analysis supports this idea by reporting no effect of smoking on the concentration of 8-isoprostane in a healthy population (*See part 4*).

In addition, we observed no significant intra-individual difference in oxidative potential originating from the alveolar or total airways part. Nevertheless, with the current material used – IR-based capnometer valve –, it is still difficult to distinguish completely the origin of exhaled air collected in the bag (airways vs. alveolar region). It is also worth noting that the deuterated water solution could not be used in the pilot field study, since this development was not yet achieved. We used Milli-Q water, i.e., water filtered through a resin and subjected to UV treatment, which can bring oxidizing species into the solution and distort the results. For the rest of the study – out from the scope of the present thesis – the deutered water was used as optimized air collection medium. Moreover, the correct way of calculating the value of OPEA is still under investigation, the dependence of the value of ambient exhaled air (especially high ambient OP levels) can have a greater impact on total airways compared to alveolar exhaled air. For this reason, the analysis of the ambient OP should be considered as a quality factor to evaluate the corresponding calculated OPEA.
In view of these preliminary results, we believe that OPEA is not a relevant biomarker of exposure – in the context of underground metro station – since physiological repairing mechanism usually enable fast redox homeostasis recovery in the lung. Further study is currently dedicated to establish the reference levels of OPEA in the healthy population (SHeS study). In the near future, we will evaluate the ability of OPEA metric to predict acute/chronic inflammation status occurring for a series of lung diseases (e.g., COPD, asthma, interstitial lung disease) [215].

In view of the results obtained during the pilot study, we decided to restrain to MDA and OPEA as biomarkers of oxidative stress for the large epidemiological study including about 300 volunteers belonging to these three professional groups is in progress in the same company.

4. Strengths of the pilot study

Our pilot study has several strengths. This study implied a multidisciplinary Franco-Swiss collaboration between a university research center and a big company (RATP), involving a

multitude of professions varying from occupational physicians, pulmonologist, toxicologists, epidemiologists, chemists, to occupational hygienists. This is the first extensive study on RATP workers' health exploring the relation between PM fractions in underground indoor air (which are quite singular) and early physio-pathological effects while checking the possible factors that could influence this relationship, such as health status and lifestyle habits (via the administration of an exhaustive questionnaire). This study design (twice a day, two weeks) prevents a large inter-individual variability that may predominate over the inter-individual variability of metabolite levels due to the small number of participants (no more participants was included because of the diversity of exposure metrics and measurements and devices available). We measure a large number of exposure metrics (*see part 3*) and a great diversity of exhaled air biomarkers including novelties such as OPEA. The use of exhaled air as non-invasive metric to give direct access to the lung as a target organ to represent oxidative stress locally. In contrast to urinary biomarkers, exhaled biomarkers are not yet routinely used in occupational medicine and research, the study is therefore of great importance for their validation and development. We use analytical chemistry validated methods for the analysis of these different metabolites. This is a prerequisite for proposing EBC as a relevant matrix for future clinical studies [220].

5. Limitations of the pilot study

The pilot study has some limitations. The results have still to be considered cautiously and require larger studies to clarify their significance as the station agents group consisted only of women which could create a gender effect.

Finally, to have insight into the actual functional status of the lungs, an approach combining multiple stress oxidative biomarkers in EBC is mandatory. Oxidative stress biomarkers are not disease-specific and can influenced by other individual, behavioral or environmental factors. For example, MDA formation is not specific to free radical-induced oxidation of lipids [408]. A characteristic "fingerprint" of different oxidative stress biomarkers could be helpful to diagnose COPD disease [181]. COPD therapies may also impose a characteristic effect on these biomarkers, which may improve the specificity of treatment in the future. The absence of information on the concentration of 8-OHdG and 8-isoprostane could constitute an important limitation for future studies.

PERSPECTIVES AND FUTURE WORKS

Future research should aim to:

- Improve the method for quantifying 8-OHdG and 8-isoprostane for use in the field (quantification by Orbitrap detection is currently being studied);
- Investigate the association between concentrations of oxidative stress biomarkers in EBC with urine;
- Assess the level of oxidative stress biomarkers if the RATP personal protective equipment is used and working habits are modified;
- Standardize via an international consensus the methods currently used for EBC. Presently, too many different sampling devices and techniques are used, rendering comparison between studies difficult at present;
- Valorize the data from the large epidemiological field study. This study will give insight on the diagnostic relevance of the biomarkers pre-selected in the pilot study for diagnosis of COPD;
- Disseminate to the RATP workers (RATP report and article in French) the results tables (part 3) that is a comprehensive review of the exposure metrics in relation to the OEL-8h or reference values in the literature;
- Propose, in the foreseeable future, the use of the most promising oxidative stress biomarkers for the diagnosis or accompanying the diagnosis of COPD at CliniMARK (EU COST Action CA16113)

CONCLUSION

This research project allowed us to examine four OS biomarkers measured in exhaled air though the series of experimental and epidemiological studies in view of the early COPD detection.

Precisely, we developed an optimized LC-ESI-MS/MS method for MDA in EBC. The developed method presented good performances despite a contamination in procedural blanks. The calculation of uncertainty has elucidated any concerns about the utilization of the method in studies focusing on comparing different volunteers or groups of subjects.
We also developed a robust method for simultaneous detection of 8-isoprostane and 8-OHdG in EBC. Finally, we performed great optical improvements on the OPEA device, making the approach particularly interesting for implementation in epidemiological settings.

We then proposed to apply these methods in epidemiological pilot study focusing on exploring the relation between three PM fractions in underground indoor air and early physio-pathological effects. We were able to detect MDA in all the EBC samples, but at very low concentrations. We observed varying degrees of association between MDA and other exposure metrics.
Concerning 8-OHdG/8-isoprostane, all results were under LOD. We suspected that the lack of detection for these biomarkers is due to factors affecting the EBC collection. Further experimental studies to standardize EBC collection devices and round robin analysis to check if analyses performed independently in different laboratories could lead to the same results for analysis of 8-OHdG and 8-isoprostane, are suitable.
Concerning the OPEA metric we observed no correlation with any of the measured exposure variables. OPEA relationship with respiratory diseases, namely the COPD is under investigation.

In conclusion, MDA seems to be a relevant biomarker of exposure while OPEA would be particularly suitable – as stated by earlier study [215] – to predict inflammatory exacerbation episodes or early diagnosis of inflammatory lung diseases. Future studies might be conducted in order to address such hypotheses and determine for these biomarkers the pertinent field of use.

VALORIZATION AND COMMUNICATION OF THE RESULTS

This work has been the subject of numerous publications.

1-Scientific articles signed as the first author

1. **Hemmendinger, M.**; Sauvain, J.; Hopf, N.; Wild, P.; Suárez, G.; Guseva Canu, I. , Challenges in quantifying 8-OHdG and 8-isoprostane in exhaled breath condensate. Antioxidants 2022, 11, 830.

2. **Hemmendinger, M.;** Sauvain, J.; Hopf, N.; Wild, P.; Suárez, G.; Guseva Canu, I., Method Validation and Characterization of the Associated Uncertainty for Malondialdehyde Quantification in Exhaled Breath Condensate. Antioxidants 2021, 10, 1661.

3. **Hemmendinger, M.;** Wild, P.; Shoman, Y.; Graille, M.; Bergamaschi, E.; Hopf, N.; Guseva Canu, I., Reference ranges of oxidative stress biomarkers selected for non-invasive biological surveillance of nanotechnology workers: Study protocol and meta-analysis results for 8-OHdG in exhaled breath condensate. Toxicol Lett 2020, 327, 41-47.

2- Scientific articles signed as co-author

1. Goekce, S.; Concha-Lozano, N.; Sauvain, J.; **Hemmendinger, M.;** Portela, A.; Sergent, E.; Andujar, P.; Pairon, J.-C.; Wild, P.; Suárez, G., Multiscattering-enhanced absorbance to enable the sensitive analysis of extremely diluted biological samples: Determination of oxidative potential in exhaled air. Medicine in Novel Technology and Devices 2022, 100120.

2. Sauvain, J. J.; **Hemmendinger, M**.; Suárez, G.; Creze, C.; Hopf, N. B.; Jouannique, V.; Debatisse, A.; Pralong, J. A.; Wild, P.; Canu, I. G., Malondialdehyde and anion patterns in exhaled breath condensate among subway workers. Part Fibre Toxicol 2022, 19 (1), 16.

3. Rayana, T.; **Hemmendinger, M.;** Crézé, C.; Wild, P.; Sauvain, J.; Suárez, G.; Besançon, S.; Méthy, N.; Sakthithasan, K.; Carillo, G.; Debatisse, A.; Jouannique, V.; Guinhouya, B. C.; Guseva Canu, I., Analyse exploratoire des mesures de particules

ultrafines en temps réel dans des enceintes ferroviaires souterraines de transport public. Archives des Maladies Professionnelles et de l'Environnement 2022.

4. Guseva Canu, I.; **Hemmendinger, M.;** Sauvain, J. J.; Suarez, G.; Hopf, N. B.; Pralong, J. A.; Ben Rayana, T.; Besançon, S.; Sakthithasan, K.; Jouannique, V.; Debatisse, A., Respiratory Disease Occupational Biomonitoring Collaborative Project (ROBoCoP): A longitudinal pilot study and implementation research in the Parisian transport company. J Occup Med Toxicol 2021, 16 (1), 22.

5. Guseva Canu, I.; Crézé, C.; **Hemmendinger, M.;** Ben Rayana, T.; Besançon, S.; Jouannique, V.; Debatisse, A.; Wild, P.; Sauvain, J. J.; Suárez, G.; Hopf, N. B., Particle and metal exposure in Parisian subway: Relationship between exposure biomarkers in air, exhaled breath condensate, and urine. Int J Hyg Environ Health 2021, 237, 113837.

6. Shoman, Y.; Wild, P.; **Hemmendinger, M.;** Graille, M.; Sauvain, J.-J.; Hopf, N. B.; Guseva Canu, I., Reference Ranges of 8-Isoprostane Concentrations in Exhaled Breath Condensate (EBC): A Systematic Review and Meta-Analysis. Int J Mol Sci 2020, 21 (11), 3822.

7. Graille, M.; Wild, P.; Sauvain, J. J.; **Hemmendinger, M.;** Guseva Canu, I.; Hopf, N. B., Urinary 8-isoprostane as a biomarker for oxidative stress. A systematic review and meta-analysis. Toxicol Lett 2020, 328, 19-27.

8. Graille, M.; Wild, P.; Sauvain, J.-J.; **Hemmendinger, M**.; Guseva Canu, I.; Hopf, N. B., Urinary 8-OHdG as a Biomarker for Oxidative Stress: A Systematic Literature Review and Meta-Analysis. Int J Mol Sci 2020, 21 (11), 3743.

This work has been the subject of numerous communications.

1- Oral Presentation

1. M. Hemmendinger, P. Wild, M. Graille, N. Hopf, I. Guseva Canu **Individual variability in oxidative stress biomarkers in exhaled breath condensate in a adult population** ISEE Young 2021, 17-18 February 2021, Virtual conference.

2. M. Hemmendinger, N. Sambiagio, S. Goekce; N. Concha-lozano, I. Guseva Canu, G. Suarez, J.J. Sauvain **Non-invasive sensitive method for oxidative stress**

quantification in exhaled air Cost CliniMARK training school: approaches for biomarker discovery and validation, 23-27 September 2019, Spetses, Greece

3. M. Hemmendinger, N. Sambiagio, I Guseva Canu, G. Suarez, J.J. Sauvain **Simultaneous analysis of a panel of three biomarkers of oxidative stress in exhaled breath condensate** 11th International Symposium on Biological Monitoring in Occupational and Environmental Health (ISBM-11). 27-30 August 2019, Leuven. Belgium

4. M. Hemmendinger, S. Goekce; N. Concha-lozano, I. Guseva Canu, J.J. Sauvain, G. Suarez **Fast and noninvasive determination of exhaled air oxidative potential occurring in the lung** 11th International Symposium on Biological Monitoring in Occupational and Environmental Health (ISBM-11). 27-30 August 2019, Leuven. Belgium

5. M. Hemmendinger, N. Sambiagio, S. Goekce; N. Concha-lozano, I. Guseva Canu, G. Suarez, J.J. Sauvain. **Optimization of methods for the detection of oxidative stress markers in exhaled air.** 5th CliniMark meeting - workshop – March 29th 2019, Basel, Switzerland.

2- Poster presentation

1. M. Hemmendinger, M. Graille, T. Ben Rayana, N. Hopf, J.J. Sauvain, G. Suárez, I. Guseva Canu **Study of inter- and intra-individual variability in the measurement of oxidative stress biomarkers for validation for biomonitoring in environmental and occupational health** 19ème colloque ADEREST, 14-15 November 2019, Toulouse, France

2. M. Hemmendinger, N. Sambiagio, S. Goekce; N. Concha-lozano, I. Guseva Canu, G. Suarez, J.J. Sauvain **Non-invasive sensitive method for oxidative stress quantification in exhaled air** Cost CliniMARK training school: approaches for biomarker discovery and validation, 23-27 September 2019, Spetses, Greece

3. M. Hemmendinger, P. Wild, M. Graille, N. Hopf, I. Guseva Canu **Individual variability in oxidative stress biomarkers in exhaled breath condensate in a healthy adult population: preliminary results for 8-OHdG** 11th International Symposium on

Biological Monitoring in Occupational and Environmental Health (ISBM-11). 27-30 August 2019, Leuven. Belgium

4. M. Graille, P. Wild, M. Hemmendinger, I. Guseva Canu, N. Hopf **Between-study variability in oxidative stress biomarker in urine in adult populations - isoprostanes** 11th International Symposium on Biological Monitoring in Occupational and Environmental Health (ISBM-11). 27-30 August 2019, Leuven. Belgium

5. M. Hemmendinger, N. Sambiagio, S. Goekce; N. Concha-lozano, I. Guseva Canu, G. Suarez, J.J. Sauvain. **Non-invasive sensitive method for oxidative stress quantification in exhaled air.** International conference Environmental and Occupational Health Aspects Related to Nano- and Ultrafine Particulate Matter. 3-6 June 2019, Loen, Norvay. http://eohnano.com

3- Others

- RATP movie and official report

- Technical support on another project : Guseva Canu, I.; Suárez, G.; Sauvain, J.; Hopf, N.; Crézé, C.; **Hemmendinger, M.;** Fito, C.; Squillacioti, G.; Ghelli, F.; Buglisi, M.; Garzaro, G.; Bergamaschi, E., Implementation of a harmonized approach for monitoring exposure to engineered and incidental nanoparticles and their potential health effects: First results from the EU-LIFE project NanoExplore. Safety and Health at Work 2022, 13, S30-S31.

REFERENCES

1. WHO. The top 10 causes of death. **2010**.
2. Murray, C.J.; Atkinson, C.; Bhalla, K.; Birbeck, G.; Burstein, R.; Chou, D.; Dellavalle, R.; Danaei, G.; Ezzati, M.; Fahimi, A.; et al. The state of US health, 1990-2010: burden of diseases, injuries, and risk factors. *Jama* **2013**, *310*, 591-608, doi:10.1001/jama.2013.13805.
3. Blanco, I.; Diego, I.; Bueno, P.; Fernández, E.; Casas-Maldonado, F.; Esquinas, C.; Soriano, J.B.; Miravitlles, M. Geographical distribution of COPD prevalence in Europe, estimated by an inverse distance weighting interpolation technique. *International journal of chronic obstructive pulmonary disease* **2017**, *13*, 57-67, doi:10.2147/COPD.S150853.
4. (FIRS), E.R.S.o.b.o.t.F.o.I.R.S. The Global Impact of Respiratory Disease, second edition. Available online: https://www.who.int/gard/publications/The_Global_Impact_of_Respiratory_Disease.pdf (accessed on 23th March).
5. Bridevaux, P.O.; Probst-Hensch, N.M.; Schindler, C.; Curjuric, I.; Felber Dietrich, D.; Braendli, O.; Brutsche, M.; Burdet, L.; Frey, M.; Gerbase, M.W.; et al. Prevalence of airflow obstruction in smokers and never-smokers in Switzerland. *European Respiratory Journal* **2010**, *36*, 1259, doi:10.1183/09031936.00004110.
6. Chen, J.; Hoek, G. Long-term exposure to PM and all-cause and cause-specific mortality: A systematic review and meta-analysis. *Environ Int* **2020**, *143*, 105974, doi:10.1016/j.envint.2020.105974.
7. Vestbo, J.; Hurd, S.S.; Agustí, A.G.; Jones, P.W.; Vogelmeier, C.; Anzueto, A.; Barnes, P.J.; Fabbri, L.M.; Martinez, F.J.; Nishimura, M.; et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* **2013**, *187*, 347-365, doi:10.1164/rccm.201204-0596PP.
8. Hogg, J.C.; Timens, W. The pathology of chronic obstructive pulmonary disease. *Annu Rev Pathol* **2009**, *4*, 435-459, doi:10.1146/annurev.pathol.4.110807.092145.
9. Brusselle, G.G.; Joos, G.F.; Bracke, K.R. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* **2011**, *378*, 1015-1026, doi:10.1016/s0140-6736(11)60988-4.
10. Wu, J.; Li, X.; Qin, Y.; Cheng, J.; Hao, G.; Jin, R.; Zhu, C. Jinwei Tang modulates HDAC2 expression in a rat model of COPD. *Exp Ther Med* **2018**, *15*, 2604-2610, doi:10.3892/etm.2018.5707.
11. Kinnula, V.L.; Ishikawa, N.; Bergmann, U.; Ohlmeier, S. Proteomic approaches for studying human parenchymal lung diseases. *Expert Review of Proteomics* **2009**, *6*, 619-629, doi:10.1586/epr.09.80.
12. Chen, W.; Thomas, J.; Sadatsafavi, M.; FitzGerald, J.M. Risk of cardiovascular comorbidity in patients with chronic obstructive pulmonary disease: a systematic review and meta-analysis. *Lancet Respir Med* **2015**, *3*, 631-639, doi:10.1016/s2213-2600(15)00241-6.
13. Donaldson, G.C.; Hurst, J.R.; Smith, C.J.; Hubbard, R.B.; Wedzicha, J.A. Increased risk of myocardial infarction and stroke following exacerbation of COPD. *Chest* **2010**, *137*, 1091-1097, doi:10.1378/chest.09-2029.
14. Feary, J.R.; Rodrigues, L.C.; Smith, C.J.; Hubbard, R.B.; Gibson, J.E. Prevalence of major comorbidities in subjects with COPD and incidence of myocardial infarction and stroke: a comprehensive analysis using data from primary care. *Thorax* **2010**, *65*, 956-962, doi:10.1136/thx.2009.128082.
15. Papaioannou, O.; Karampitsakos, T.; Barbayianni, I.; Chrysikos, S.; Xylourgidis, N.; Tzilas, V.; Bouros, D.; Aidinis, V.; Tzouvelekis, A. Metabolic Disorders in Chronic Lung Diseases. *Front Med (Lausanne)* **2018**, *4*, 246-246, doi:10.3389/fmed.2017.00246.
16. Chen, X.; Wang, T.; Qiu, X.; Que, C.; Zhang, H.; Zhang, L.; Zhu, T. Susceptibility of individuals with chronic obstructive pulmonary disease to air pollution exposure in Beijing, China: A case-

control panel study (COPDB). *Science of The Total Environment* **2020**, *717*, 137285, doi:https://doi.org/10.1016/j.scitotenv.2020.137285.

17. Sunyer, J.; Schwartz, J.; Tobías, A.; Macfarlane, D.; Garcia, J.; Antó, J.M. Patients with chronic obstructive pulmonary disease are at increased risk of death associated with urban particle air pollution: a case-crossover analysis. *Am J Epidemiol* **2000**, *151*, 50-56, doi:10.1093/oxfordjournals.aje.a010121.
18. Jensen, M.T.; Marott, J.L.; Lange, P.; Vestbo, J.; Schnohr, P.; Nielsen, O.W.; Jensen, J.S.; Jensen, G.B. Resting heart rate is a predictor of mortality in COPD. *European Respiratory Journal* **2013**, *42*, 341, doi:10.1183/09031936.00072212.
19. Sin, D.D.; Man, J.P.; Man, S.F. The risk of osteoporosis in Caucasian men and women with obstructive airways disease. *Am J Med* **2003**, *114*, 10-14, doi:10.1016/s0002-9343(02)01297-4.
20. Wouters, E.F.; Creutzberg, E.C.; Schols, A.M. Systemic effects in COPD. *Chest* **2002**, *121*, 127s-130s, doi:10.1378/chest.121.5_suppl.127s.
21. Fry, J.S.; Hamling, J.S.; Lee, P.N. Systematic review with meta-analysis of the epidemiological evidence relating FEV1decline to lung cancer risk. *BMC Cancer* **2012**, *12*, 498, doi:10.1186/1471-2407-12-498.
22. Vogiatzis, I.; Terzis, G.; Nanas, S.; Stratakos, G.; Simoes, D.C.; Georgiadou, O.; Zakynthinos, S.; Roussos, C. Skeletal muscle adaptations to interval training in patients with advanced COPD. *Chest* **2005**, *128*, 3838-3845, doi:10.1378/chest.128.6.3838.
23. Papi, A.; Luppi, F.; Franco, F.; Fabbri, L.M. Pathophysiology of exacerbations of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* **2006**, *3*, 245-251, doi:10.1513/pats.200512-125SF.
24. Seemungal, T.A.; Donaldson, G.C.; Bhowmik, A.; Jeffries, D.J.; Wedzicha, J.A. Time course and recovery of exacerbations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **2000**, *161*, 1608-1613, doi:10.1164/ajrccm.161.5.9908022.
25. Thomas, M.; Decramer, M.; O'Donnell, D.E. No room to breathe: the importance of lung hyperinflation in COPD. *Primary Care Respiratory Journal* **2013**, *22*, 101-111, doi:10.4104/pcrj.2013.00025.
26. Seemungal, T.A.; Donaldson, G.C.; Paul, E.A.; Bestall, J.C.; Jeffries, D.J.; Wedzicha, J.A. Effect of exacerbation on quality of life in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **1998**, *157*, 1418-1422, doi:10.1164/ajrccm.157.5.9709032.
27. Miravitlles, M.; Mayordomo, C.; Artés, M.; Sánchez-Agudo, L.; Nicolau, F.; Segú, J.L. Treatment of chronic obstructive pulmonary disease and its exacerbations in general practice. EOLO Group. Estudio Observacional de la Limitación Obstructiva al Flujo aEreo. *Respir Med* **1999**, *93*, 173-179, doi:10.1016/s0954-6111(99)90004-5.
28. Soler-Cataluña, J.J.; Martínez-García, M.A.; Román Sánchez, P.; Salcedo, E.; Navarro, M.; Ochando, R. Severe acute exacerbations and mortality in patients with chronic obstructive pulmonary disease. *Thorax* **2005**, *60*, 925-931, doi:10.1136/thx.2005.040527.
29. Connors, A.F., Jr.; Dawson, N.V.; Thomas, C.; Harrell, F.E., Jr.; Desbiens, N.; Fulkerson, W.J.; Kussin, P.; Bellamy, P.; Goldman, L.; Knaus, W.A. Outcomes following acute exacerbation of severe chronic obstructive lung disease. The SUPPORT investigators (Study to Understand Prognoses and Preferences for Outcomes and Risks of Treatments). *Am J Respir Crit Care Med* **1996**, *154*, 959-967, doi:10.1164/ajrccm.154.4.8887592.
30. Groenewegen, K.H.; Schols, A.M.; Wouters, E.F. Mortality and mortality-related factors after hospitalization for acute exacerbation of COPD. *Chest* **2003**, *124*, 459-467, doi:10.1378/chest.124.2.459.
31. Almagro, P.; Calbo, E.; Ochoa de Echagüen, A.; Barreiro, B.; Quintana, S.; Heredia, J.L.; Garau, J. Mortality after hospitalization for COPD. *Chest* **2002**, *121*, 1441-1448, doi:10.1378/chest.121.5.1441.

32. Organization, W.H. Projections of mortality and causes of death, 2016 and 2060, http://www.who.int/healthinfo/global_burden_disease/projections/en/. Available online: (accessed on 31/01).
33. Mercado, N.; Ito, K.; Barnes, P.J. Accelerated ageing of the lung in COPD: new concepts. *Thorax* **2015**, *70*, 482-489, doi:10.1136/thoraxjnl-2014-206084.
34. Foreman, M.G.; Zhang, L.; Murphy, J.; Hansel, N.N.; Make, B.; Hokanson, J.E.; Washko, G.; Regan, E.A.; Crapo, J.D.; Silverman, E.K.; et al. Early-onset chronic obstructive pulmonary disease is associated with female sex, maternal factors, and African American race in the COPDGene Study. *American journal of respiratory and critical care medicine* **2011**, *184*, 414-420, doi:10.1164/rccm.201011-1928OC.
35. Silverman, E.K.; Weiss, S.T.; Drazen, J.M.; Chapman, H.A.; Carey, V.; Campbell, E.J.; Denish, P.; Silverman, R.A.; Celedon, J.C.; Reilly, J.J.; et al. Gender-related differences in severe, early-onset chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **2000**, *162*, 2152-2158, doi:10.1164/ajrccm.162.6.2003112.
36. Silva, G.E.; Sherrill, D.L.; Guerra, S.; Barbee, R.A. Asthma as a risk factor for COPD in a longitudinal study. *Chest* **2004**, *126*, 59-65, doi:10.1378/chest.126.1.59.
37. Stoller, J.K.; Aboussouan, L.S. Alpha1-antitrypsin deficiency. *Lancet* **2005**, *365*, 2225-2236, doi:10.1016/s0140-6736(05)66781-5.
38. Kohansal, R.; Martinez-Camblor, P.; Agustí, A.; Buist, A.S.; Mannino, D.M.; Soriano, J.B. The natural history of chronic airflow obstruction revisited: an analysis of the Framingham offspring cohort. *Am J Respir Crit Care Med* **2009**, *180*, 3-10, doi:10.1164/rccm.200901-0047OC.
39. Forey, B.A.; Thornton, A.J.; Lee, P.N. Systematic review with meta-analysis of the epidemiological evidence relating smoking to COPD, chronic bronchitis and emphysema. *BMC Pulm Med* **2011**, *11*, 36-36, doi:10.1186/1471-2466-11-36.
40. Yin, P.; Jiang, C.Q.; Cheng, K.K.; Lam, T.H.; Lam, K.H.; Miller, M.R.; Zhang, W.S.; Thomas, G.N.; Adab, P. Passive smoking exposure and risk of COPD among adults in China: the Guangzhou Biobank Cohort Study. *Lancet* **2007**, *370*, 751-757, doi:10.1016/s0140-6736(07)61378-6.
41. Townend, J.; Minelli, C.; Mortimer, K.; Obaseki, D.O.; Al Ghobain, M.; Cherkaski, H.; Denguezli, M.; Gunesekera, K.; Hafizi, H.; Koul, P.A.; et al. The association between chronic airflow obstruction and poverty in 12 sites of the multinational BOLD study. *Eur Respir J* **2017**, *49*, doi:10.1183/13993003.01880-2016.
42. Hinds, W.C. *Aerosol Technology, Properties, Behaviour, and Measurement of Airborne Particles. John Wiley & Sons Inc., New York.* **1999**.
43. Commission, E. Directive 2008/50/EC of the European Parliament and of the council of 21 May 2008 on ambient air quality and cleaner for Europe. *Offical Journal of the European Union, L152,1-44* **2008**.
44. Muhlfeld, C.; Rothen-Rutishauser, B.; Blank, F.; Vanhecke, D.; Ochs, M.; Gehr, P. Interactions of nanoparticles with pulmonary structures and cellular responses. *American journal of physiology. Lung cellular and molecular physiology* **2008**, *294*, L817-829, doi:10.1152/ajplung.00442.2007.
45. Eisner, M.D.; Anthonisen, N.; Coultas, D.; Kuenzli, N.; Perez-Padilla, R.; Postma, D.; Romieu, I.; Silverman, E.K.; Balmes, J.R. An official American Thoracic Society public policy statement: Novel risk factors and the global burden of chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine* **2010**, *182*, 693-718, doi:10.1164/rccm.200811-1757ST.
46. Postma, D.S.; Bush, A.; van den Berge, M. Risk factors and early origins of chronic obstructive pulmonary disease. *Lancet (London, England)* **2015**, *385*, 899-909, doi:10.1016/s0140-6736(14)60446-3.
47. Caillaud, D.; Lemoigne, F.; Carré, P.; Escamilla, R.; Chanez, P.; Burgel, P.-R.; Court-Fortune, I.; Jebrak, G.; Pinet, C.; Perez, T.; et al. Association between occupational exposure and the

clinical characteristics of COPD. *BMC Public Health* **2012**, *12*, 302, doi:10.1186/1471-2458-12-302.
48. Seaton, A.; Cherrie, J.; Dennekamp, M.; Donaldson, K.; Hurley, J.F.; Tran, C.L. The London Underground: dust and hazards to health. *Occup Environ Med* **2005**, *62*, 355-362, doi:10.1136/oem.2004.014332.
49. Schraufnagel, D.E. The health effects of ultrafine particles. *Experimental & Molecular Medicine* **2020**, *52*, 311-317, doi:10.1038/s12276-020-0403-3.
50. Bayer-Oglesby, L.; Grize, L.; Gassner, M.; Takken-Sahli, K.; Sennhauser, F.H.; Neu, U.; Schindler, C.; Braun-Fahrlander, C. Decline of ambient air pollution levels and improved respiratory health in Swiss children. *Environmental health perspectives* **2005**, *113*, 1632-1637, doi:10.1289/ehp.8159.
51. Schindler, C.; Keidel, D.; Gerbase, M.W.; Zemp, E.; Bettschart, R.; Brandli, O.; Brutsche, M.H.; Burdet, L.; Karrer, W.; Knopfli, B.; et al. Improvements in PM10 exposure and reduced rates of respiratory symptoms in a cohort of Swiss adults (SAPALDIA). *American journal of respiratory and critical care medicine* **2009**, *179*, 579-587, doi:10.1164/rccm.200803-388OC.
52. WHO. Health aspects of air pollution: Results from the WHO project systematic review of health aspects of air pollution in Europe. **2004**.
53. Schwarze, P.E.; Ovrevik, J.; Lag, M.; Refsnes, M.; Nafstad, P.; Hetland, R.B.; Dybing, E. Particulate matter properties and health effects: consistency of epidemiological and toxicological studies. *Human & experimental toxicology* **2006**, *25*, 559-579, doi:10.1177/096032706072520.
54. Pope, C.A.; Dockery, D.W. Health Effects of Fine Particulate Air Pollution: Lines that Connect. *Journal of the Air & Waste Management Association* **2006**, *56*, 709-742, doi:10.1080/10473289.2006.10464485.
55. Organization, W.h. One third of global air pollution deaths in Asia Pacific. Available online: https://www.who.int/westernpacific/news/item/02-05-2018-one-third-of-global-air-pollution-deaths-in-asia-pacific (accessed on 23th march).
56. Brauer, M.; Amann, M.; Burnett, R.T.; Cohen, A.; Dentener, F.; Ezzati, M.; Henderson, S.B.; Krzyzanowski, M.; Martin, R.V.; Van Dingenen, R.; et al. Exposure assessment for estimation of the global burden of disease attributable to outdoor air pollution. *Environmental science & technology* **2012**, *46*, 652-660, doi:10.1021/es2025752.
57. WHO. *WHO global air quality guidelines. Particulate matter (PM2.5 and PM10), ozone, nitrogen dioxide,*

sulfur dioxide and carbon monoxide.; Geneva, Switzerland, 2021; p. 300.
58. Nordgren, T.M.; Charavaryamath, C. Agriculture Occupational Exposures and Factors Affecting Health Effects. *Curr Allergy Asthma Rep* **2018**, *18*, 65-65, doi:10.1007/s11882-018-0820-8.
59. Stoleski, S.; Minov, J.; Karadzinska-Bislimovska, J.; Mijakoski, D. Chronic obstructive pulmonary disease in never-smoking dairy farmers. *Open Respir Med J* **2015**, *9*, 59-66, doi:10.2174/1874306401509010059.
60. Koh, D.H.; Kim, J.I.; Kim, K.H.; Yoo, S.W.; the Korea Welders Cohort, G. Welding fume exposure and chronic obstructive pulmonary disease in welders. *Occupational Medicine* **2015**, *65*, 72-77, doi:10.1093/occmed/kqu136.
61. Grahn, K.; Gustavsson, P.; Andersson, T.; Lindén, A.; Hemmingsson, T.; Selander, J.; Wiebert, P. Occupational exposure to particles and increased risk of developing chronic obstructive pulmonary disease (COPD): A population-based cohort study in Stockholm, Sweden. *Environmental Research* **2021**, *200*, 111739, doi:https://doi.org/10.1016/j.envres.2021.111739.
62. Nieuwenhuijsen, M.J.; Gómez-Perales, J.E.; Colvile, R.N. Levels of particulate air pollution, its elemental composition, determinants and health effects in metro systems. *Atmospheric Environment* **2007**, *41*, 7995-8006, doi:https://doi.org/10.1016/j.atmosenv.2007.08.002.

63. Ripanucci, G.; Grana, M.; Vicentini, L.; Magrini, A.; Bergamaschi, A. Dust in the underground railway tunnels of an Italian town. *Journal of occupational and environmental hygiene* **2006**, *3*, 16-25, doi:10.1080/15459620500444004.
64. Loxham, M.; Cooper, M.J.; Gerlofs-Nijland, M.E.; Cassee, F.R.; Davies, D.E.; Palmer, M.R.; Teagle, D.A.H. Physicochemical characterization of airborne particulate matter at a mainline underground railway station. *Environ Sci Technol* **2013**, *47*, 3614-3622, doi:10.1021/es304481m.
65. ANSES. Rapport d'expertise collective : Pollution chimique de l'air des enceintes de transports ferroviaires souterrains et risques sanitaires associés chez les travailleurs **2015**.
66. Kam, W.; Delfino, R.J.; Schauer, J.J.; Sioutas, C. A comparative assessment of PM2.5 exposures in light-rail, subway, freeway, and surface street environments in Los Angeles and estimated lung cancer risk. *Environmental Science: Processes & Impacts* **2013**, *15*, 234-243, doi:10.1039/C2EM30495C.
67. Kim, H.H.; Lim, Y.W.; Jeon, J.M.; Kim, T.H.; Lee, G.W.; Lee, W.S.; Lim, J.Y.; Shin, D.C.; Yang, J.Y. Indoor exposure and health risk of polycyclic aromatic hydrocarbons (PAHs) in public facilities, Korea. *Asian Journal of Atmospheric Environment* **2013**, *7*, 72-84, doi:10.5572/ajae.2013.7.2.072.
68. Institute, H.E. HEI - Diesel Exhaust: Critical Analysis of Emissions, Exposure, and Health Effects. *Special Report* **1995**.
69. Loxham, M.; Woo, J.; Singhania, A.; Smithers, N.P.; Yeomans, A.; Packham, G.; Crainic, A.M.; Cook, R.B.; Cassee, F.R.; Woelk, C.H.; et al. Upregulation of epithelial metallothioneins by metal-rich ultrafine particulate matter from an underground railway. *Metallomics* **2020**, *12*, 1070-1082, doi:10.1039/d0mt00014k.
70. Wen, Y.; Leng, J.; Shen, X.; Han, G.; Sun, L.; Yu, F. Environmental and health effects of ventilation in subway stations: a literature review. *International journal of environmental research and public health* **2020**, *17*, 1084.
71. Health, H.C.o.P. Pollution par les particules dans l'air ambiant **2012**.
72. Gustavsson, P.; Bigert, C.; Pollan, M. Incidence of lung cancer among subway drivers in Stockholm. *American journal of industrial medicine* **2008**, *51*, 545-547, doi:10.1002/ajim.20584.
73. Grass, D.S.; Ross, J.M.; Family, F.; Barbour, J.; James Simpson, H.; Coulibaly, D.; Hernandez, J.; Chen, Y.; Slavkovich, V.; Li, Y.; et al. Airborne particulate metals in the New York City subway: a pilot study to assess the potential for health impacts. *Environmental research* **2010**, *110*, 1-11, doi:10.1016/j.envres.2009.10.006.
74. Bigert, C.; Alderling, M.; Svartengren, M.; Plato, N.; de Faire, U.; Gustavsson, P. Blood markers of inflammation and coagulation and exposure to airborne particles in employees in the Stockholm underground. *Occupational and environmental medicine* **2008**, *65*, 655-658, doi:10.1136/oem.2007.038273.
75. Seaton, A.; Cherrie, J.; Dennekamp, M.; Donaldson, K.; Hurley, J.F.; Tran, C.L. The London Underground: dust and hazards to health. *Occupational and environmental medicine* **2005**, *62*, 355-362, doi:10.1136/oem.2004.014332.
76. Lindbom, J.; Gustafsson, M.; Blomqvist, G.; Dahl, A.; Gudmundsson, A.; Swietlicki, E.; Ljungman, A.G. Wear particles generated from studded tires and pavement induces inflammatory reactions in mouse macrophage cells. *Chemical research in toxicology* **2007**, *20*, 937-946, doi:10.1021/tx700018z.
77. Li, N.; Kim, S.; Wang, M.; Froines, J.; Sioutas, C.; Nel, A. Use of a stratified oxidative stress model to study the biological effects of ambient concentrated and diesel exhaust particulate matter. *Inhalation toxicology* **2002**, *14*, 459-486, doi:10.1080/089583701753678571.
78. Karlsson, H.L.; Ljungman, A.G.; Lindbom, J.; Moller, L. Comparison of genotoxic and inflammatory effects of particles generated by wood combustion, a road simulator and collected from street and subway. *Toxicology letters* **2006**, *165*, 203-211, doi:10.1016/j.toxlet.2006.04.003.

79. Karlsson, H.L.; Nilsson, L.; Moller, L. Subway particles are more genotoxic than street particles and induce oxidative stress in cultured human lung cells. *Chemical research in toxicology* **2005**, *18*, 19-23, doi:10.1021/tx049723c.
80. Hulsmann, A.R.; Raatgeep, H.R.; den Hollander, J.C.; Stijnen, T.; Saxena, P.R.; Kerrebijn, K.F.; de Jongste, J.C. Oxidative epithelial damage produces hyperresponsiveness of human peripheral airways. *Am J Respir Crit Care Med* **1994**, *149*, 519-525, doi:10.1164/ajrccm.149.2.8306055.
81. Araujo, J.A.; Barajas, B.; Kleinman, M.; Wang, X.; Bennett, B.J.; Gong, K.W.; Navab, M.; Harkema, J.; Sioutas, C.; Lusis, A.J.; et al. Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. *Circulation research* **2008**, *102*, 589-596, doi:10.1161/circresaha.107.164970.
82. Buschini, A.; Cassoni, F.; Anceschi, E.; Pasini, L.; Poli, P.; Rossi, C. Urban airborne particulate: genotoxicity evaluation of different size fractions by mutagenesis tests on microorganisms and comet assay. *Chemosphere* **2001**, *44*, 1723-1736, doi:10.1016/s0045-6535(00)00550-6.
83. Healey, K.; Lingard, J.J.; Tomlin, A.S.; Hughes, A.; White, K.L.; Wild, C.P.; Routledge, M.N. Genotoxicity of size-fractionated samples of urban particulate matter. *Environmental and molecular mutagenesis* **2005**, *45*, 380-387, doi:10.1002/em.20105.
84. Nel, A.; Xia, T.; Mädler, L.; Li, N. Toxic Potential of Materials at the Nanolevel. *Science* **2006**, *311*, 622, doi:10.1126/science.1114397.
85. Oberdorster, G. Pulmonary effects of inhaled ultrafine particles. *International archives of occupational and environmental health* **2001**, *74*, 1-8, doi:10.1007/s004200000185.
86. Tao, F.; Gonzalez-Flecha, B.; Kobzik, L. Reactive oxygen species in pulmonary inflammation by ambient particulates. *Free radical biology & medicine* **2003**, *35*, 327-340, doi:10.1016/s0891-5849(03)00280-6.
87. Brown, D.M.; Wilson, M.R.; MacNee, W.; Stone, V.; Donaldson, K. Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. *Toxicology and applied pharmacology* **2001**, *175*, 191-199, doi:10.1006/taap.2001.9240.
88. Karlsson, H.L.; Holgersson, A.; Möller, L. Mechanisms related to the genotoxicity of particles in the subway and from other sources. *Chemical research in toxicology* **2008**, *21*, 726-731, doi:10.1021/tx7003568.
89. Kam, W.; Ning, Z.; Shafer, M.M.; Schauer, J.J.; Sioutas, C. Chemical Characterization and Redox Potential of Coarse and Fine Particulate Matter (PM) in Underground and Ground-Level Rail Systems of the Los Angeles Metro. *Environmental science & technology* **2011**, *45*, 6769-6776, doi:10.1021/es201195e.
90. Pellegrino, R.; Viegi, G.; Brusasco, V.; Crapo, R.O.; Burgos, F.; Casaburi, R.; Coates, A.; van der Grinten, C.P.M.; Gustafsson, P.; Hankinson, J.; et al. Interpretative strategies for lung function tests. *European Respiratory Journal* **2005**, *26*, 948, doi:10.1183/09031936.05.00035205.
91. Leah Witt; Cyrus Askin; Watto, M. COPD: Diagnosis, treatment, PFTs, and nihilism. Available online: https://thecurbsiders.com/podcast/86-copd-diagnosis-treatment-pfts-nihilism (accessed on 23th march).
92. Müllerova, H.; Maselli, D.J.; Locantore, N.; Vestbo, J.; Hurst, J.R.; Wedzicha, J.A.; Bakke, P.; Agusti, A.; Anzueto, A. Hospitalized exacerbations of COPD: risk factors and outcomes in the ECLIPSE cohort. *Chest* **2015**, *147*, 999-1007, doi:10.1378/chest.14-0655.
93. Soriano, J.B.; Lamprecht, B.; Ramírez, A.S.; Martinez-Camblor, P.; Kaiser, B.; Alfageme, I.; Almagro, P.; Casanova, C.; Esteban, C.; Soler-Cataluña, J.J.; et al. Mortality prediction in chronic obstructive pulmonary disease comparing the GOLD 2007 and 2011 staging systems: a pooled analysis of individual patient data. *Lancet Respir Med* **2015**, *3*, 443-450, doi:10.1016/s2213-2600(15)00157-5.
94. Hurst, J.R.; Vestbo, J.; Anzueto, A.; Locantore, N.; Müllerova, H.; Tal-Singer, R.; Miller, B.; Lomas, D.A.; Agusti, A.; MacNee, W.; et al. Susceptibility to Exacerbation in Chronic Obstructive Pulmonary Disease. *New England Journal of Medicine* **2010**, *363*, 1128-1138, doi:10.1056/NEJMoa0909883.

95. Bestall, J.C.; Paul, E.A.; Garrod, R.; Garnham, R.; Jones, P.W.; Wedzicha, J.A. Usefulness of the Medical Research Council (MRC) dyspnoea scale as a measure of disability in patients with chronic obstructive pulmonary disease. *Thorax* **1999**, *54*, 581-586, doi:10.1136/thx.54.7.581.
96. Jones, P.W.; Harding, G.; Berry, P.; Wiklund, I.; Chen, W.H.; Kline Leidy, N. Development and first validation of the COPD Assessment Test. *Eur Respir J* **2009**, *34*, 648-654, doi:10.1183/09031936.00102509.
97. (GOLD), G.i.f.c.o.l.d. Global Strategy for Prevention, Diagnosis and Management of COPD. **2021**.
98. Lange, P.; Halpin, D.M.; O'Donnell, D.E.; MacNee, W. Diagnosis, assessment, and phenotyping of COPD: beyond FEV_1. *Int J Chron Obstruct Pulmon Dis* **2016**, *11 Spec Iss*, 3-12, doi:10.2147/copd.S85976.
99. Regan, E.A.; Lynch, D.A.; Curran-Everett, D.; Curtis, J.L.; Austin, J.H.; Grenier, P.A.; Kauczor, H.U.; Bailey, W.C.; DeMeo, D.L.; Casaburi, R.H.; et al. Clinical and Radiologic Disease in Smokers With Normal Spirometry. *JAMA Intern Med* **2015**, *175*, 1539-1549, doi:10.1001/jamainternmed.2015.2735.
100. Seyedmehdi, S.M.; Attarchi, M.; Yazdanparast, T.; Lakeh, M.M. Quality of spirometry tests and pulmonary function changes among industrial company workers in Iran: a two-year before-and-after study following an intensive training intervention. *Prim Care Respir J* **2013**, *22*, 86-91, doi:10.4104/pcrj.2013.00018.
101. Eaton, T.; Withy, S.; Garrett, J.E.; Whitlock, R.M.L.; Rea, H.H.; Mercer, J. Spirometry in Primary Care Practice: The Importance of Quality Assurance and the Impact of Spirometry Workshops. *Chest* **1999**, *116*, 416-423, doi:https://doi.org/10.1378/chest.116.2.416.
102. Assessment, W.I.P.o.C.S.B.i.R. Validity and Validation *http://www.inchem.org/documents/ehc/ehc/ehc222.htm*. **2001**.
103. Assessment, W.I.P.o.C.S.B.a.R. Concepts and Principles *http://www.inchem.org/documents/ehc/ehc/ehc155.htm*. **1993**.
104. Mayeux, R. Biomarkers: potential uses and limitations. *NeuroRx* **2004**, *1*, 182-188, doi:10.1602/neurorx.1.2.182.
105. Ladeira, C.; Viegas, S. Human Biomonitoring – An overview on biomarkers and their application in Occupational and Environmental Health. *Biomonitoring* **2016**, *3*, doi:10.1515/bimo-2016-0003.
106. Strimbu, K.; Tavel, J.A. What are biomarkers? *Curr Opin HIV AIDS* **2010**, *5*, 463-466, doi:10.1097/COH.0b013e32833ed177.
107. Fleming, T.R.; DeMets, D.L. Surrogate End Points in Clinical Trials: Are We Being Misled? *Annals of Internal Medicine* **1996**, *125*, 605-613, doi:10.7326/0003-4819-125-7-199610010-00011.
108. Echt, D.S.; Liebson, P.R.; Mitchell, L.B.; Peters, R.W.; Obias-Manno, D.; Barker, A.H.; Arensberg, D.; Baker, A.; Friedman, L.; Greene, H.L.; et al. Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The Cardiac Arrhythmia Suppression Trial. *The New England journal of medicine* **1991**, *324*, 781-788, doi:10.1056/nejm199103213241201.
109. Perera, F.P.; Weinstein, I.B. Molecular epidemiology: recent advances and future directions. *Carcinogenesis* **2000**, *21*, 517-524, doi:10.1093/carcin/21.3.517.
110. Timbrell, J.A. Biomarkers in toxicology. *Toxicology* **1998**, *129*, 1-12, doi:10.1016/s0300-483x(98)00058-4.
111. Henderson, R.F.; Bechtold, W.E.; Bond, J.A.; Sun, J.D. The Use of Biological Markers in Toxicology. *Critical Reviews in Toxicology* **1989**, *20*, 65-82, doi:10.3109/10408448909017904.
112. Ottman, R. Gene-environment interaction: definitions and study designs. *Prev Med* **1996**, *25*, 764-770, doi:10.1006/pmed.1996.0117.
113. Biological markers in environmental health research. Committee on Biological Markers of the National Research Council. *Environ Health Perspect* **1987**, *74*, 3-9, doi:10.1289/ehp.74-1474499.

114. Petersen, R.; Thomsen, J.F.; Jørgensen, N.K.; Mikkelsen, S. Half life of chromium in serum and urine in a former plasma cutter of stainless steel. *Occup Environ Med* **2000**, *57*, 140-142, doi:10.1136/oem.57.2.140.
115. Trock, B.J. Application of biological markers in cancer environmental epidemiology. *Toxicology* **1995**, *101*, 93-98, doi:10.1016/0300-483x(95)03022-8.
116. Gleason, D.F.; Mellinger, G.T. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *J Urol* **1974**, *111*, 58-64, doi:10.1016/s0022-5347(17)59889-4.
117. Epstein, J.I.; Egevad, L.; Amin, M.B.; Delahunt, B.; Srigley, J.R.; Humphrey, P.A. The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. *Am J Surg Pathol* **2016**, *40*, 244-252, doi:10.1097/pas.0000000000000530.
118. Manno, M.; Viau, C.; Cocker, J.; Colosio, C.; Lowry, L.; Mutti, A.; Nordberg, M.; Wang, S. Biomonitoring for occupational health risk assessment (BOHRA). *Toxicology letters* **2010**, *192*, 3-16, doi:10.1016/j.toxlet.2009.05.001.
119. Stokinger, H.E.; Mountain, J.T. Test for hypersusceptibility to hemolytic chemicals. *Archives of environmental health* **1963**, *6*, 495-502, doi:10.1080/00039896.1963.10663432.
120. Comhair, S.A.; Ricci, K.S.; Arroliga, M.; Lara, A.R.; Dweik, R.A.; Song, W.; Hazen, S.L.; Bleecker, E.R.; Busse, W.W.; Chung, K.F.; et al. Correlation of systemic superoxide dismutase deficiency to airflow obstruction in asthma. *American journal of respiratory and critical care medicine* **2005**, *172*, 306-313, doi:10.1164/rccm.200502-180OC.
121. Dor, F.; Dab, W.; Empereur-Bissonnet, P.; Zmirou, D. Validity of biomarkers in environmental health studies: the case of PAHs and benzene. *Crit Rev Toxicol* **1999**, *29*, 129-168, doi:10.1080/10408449991349195.
122. Freedman, L.P.; Cockburn, I.M.; Simcoe, T.S. The Economics of Reproducibility in Preclinical Research. *PLOS Biology* **2015**, *13*, e1002165, doi:10.1371/journal.pbio.1002165.
123. Clinimark. Available online: https://clinimark.eu/clinimark.html (accessed on 31/01).
124. Aydindogan, E.; Penque, D.; Zoidakis, J. Systematic review on recent potential biomarkers of chronic obstructive pulmonary disease. *Expert Review of Molecular Diagnostics* **2019**, *19*, 37-45, doi:10.1080/14737159.2018.1559054.
125. Frantzi, M.; Latosinska, A.; Kontostathi, G.; Mischak, H. Clinical Proteomics: Closing the Gap from Discovery to Implementation. *PROTEOMICS* **2018**, *18*, 1700463, doi:https://doi.org/10.1002/pmic.201700463.
126. Repine, J.E.; Bast, A.; Lankhorst, I. Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group. *Am J Respir Crit Care Med* **1997**, *156*, 341-357, doi:10.1164/ajrccm.156.2.9611013.
127. Sies, H. Oxidative stress: oxidants and antioxidants. *Experimental Physiology* **1997**, *82*, 291-295, doi:10.1113/expphysiol.1997.sp004024.
128. Halliwell, B.; Whiteman, M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *British journal of pharmacology* **2004**, *142*, 231-255, doi:10.1038/sj.bjp.0705776.
129. Halliwell, B.a.G., J.M.C. . *Free radicals in biology and medicine. 4th Edition, Oxford University Press, New York.* **2007**.
130. Birben, E.; Sahiner, U.M.; Sackesen, C.; Erzurum, S.; Kalayci, O. Oxidative stress and antioxidant defense. *World Allergy Organ J* **2012**, *5*, 9-19, doi:10.1097/WOX.0b013e3182439613.
131. Nel, A.; Xia, T.; Madler, L.; Li, N. Toxic potential of materials at the nanolevel. *Science* **2006**, *311*, 622-627, doi:10.1126/science.1114397.
132. Lentsch, A.B.; Czermak, B.; Bless, N.; Van Rooijen, N.; Ward, P.A. Essential Role of Alveolar Macrophages in Intrapulmonary Activation of NF- ?? B. *American journal of respiratory cell and molecular biology* **1999**, *20*, 692-698, doi:10.1165/ajrcmb.20.4.3414.

133. Dalton, T.P.; Shertzer, H.G.; Puga, A. Regulation of gene expression by reactive oxygen. *Annual review of pharmacology and toxicology* **1999**, *39*, 67-101, doi:10.1146/annurev.pharmtox.39.1.67.
134. Parthasarathy, S.; Santanam, N.; Ramachandran, S.; Meilhac, O. Potential role of oxidized lipids and lipoproteins in antioxidant defense. *Free radical research* **2000**, *33*, 197-215, doi:10.1080/10715760000301381.
135. Liu, Q.; Raina, A.K.; Smith, M.A.; Sayre, L.M.; Perry, G. Hydroxynonenal, toxic carbonyls, and Alzheimer disease. *Molecular aspects of medicine* **2003**, *24*, 305-313, doi:10.1016/s0098-2997(03)00025-6.
136. Asami, S.; Manabe, H.; Miyake, J.; Tsurudome, Y.; Hirano, T.; Yamaguchi, R.; Itoh, H.; Kasai, H. Cigarette smoking induces an increase in oxidative DNA damage, 8-hydroxydeoxyguanosine, in a central site of the human lung. *Carcinogenesis* **1997**, *18*, 1763-1766, doi:10.1093/carcin/18.9.1763.
137. Andreadis, A.A.; Hazen, S.L.; Comhair, S.A.; Erzurum, S.C. Oxidative and nitrosative events in asthma. *Free radical biology & medicine* **2003**, *35*, 213-225, doi:10.1016/s0891-5849(03)00278-8.
138. Sohal, R.S.; Mockett, R.J.; Orr, W.C. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. *Free radical biology & medicine* **2002**, *33*, 575-586, doi:10.1016/s0891-5849(02)00886-9.
139. Cooke, M.S.; Lunec, J.; Evans, M.D. Progress in the analysis of urinary oxidative DNA damage. *Free radical biology & medicine* **2002**, *33*, 1601-1614, doi:10.1016/s0891-5849(02)01146-2.
140. Hollstein, M.C.; Brooks, P.; Linn, S.; Ames, B.N. Hydroxymethyluracil DNA glycosylase in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **1984**, *81*, 4003-4007, doi:10.1073/pnas.81.13.4003.
141. Gutteridge, B.H.a.J.M.C. *Free Radicals in Biology and Medicine* **2015**, doi:10.1093/acprof:oso/9780198717478.001.0001.
142. Le, N.-A. Lipoprotein-associated oxidative stress: a new twist to the postprandial hypothesis. *Int J Mol Sci* **2014**, *16*, 401-419, doi:10.3390/ijms16010401.
143. Ito, F.; Sono, Y.; Ito, T. Measurement and Clinical Significance of Lipid Peroxidation as a Biomarker of Oxidative Stress: Oxidative Stress in Diabetes, Atherosclerosis, and Chronic Inflammation. *Antioxidants (Basel)* **2019**, *8*, 72, doi:10.3390/antiox8030072.
144. Siu, G.M.; Draper, H.H. Metabolism of malonaldehyde in vivo and in vitro. *Lipids* **1982**, *17*, 349-355, doi:10.1007/bf02535193.
145. Nourooz-Zadeh, J.; Liu, E.H.; Yhlen, B.; Anggard, E.E.; Halliwell, B. F4-isoprostanes as specific marker of docosahexaenoic acid peroxidation in Alzheimer's disease. *Journal of neurochemistry* **1999**, *72*, 734-740, doi:10.1046/j.1471-4159.1999.0720734.x.
146. Roberts, L.J., 2nd; Morrow, J.D. Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation. *Cellular and molecular life sciences : CMLS* **2002**, *59*, 808-820, doi:10.1007/s00018-002-8469-8.
147. Brock, T.G. Isoprostanes. *Cayman chemical* **2012**.
148. Mueller, M.J. Isoprostane nomenclature: Inherent problems may cause setbacks for the development of the isoprostanoid field. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **2010**, *82*, 71-81, doi:https://doi.org/10.1016/j.plefa.2009.11.007.
149. Morrow, J.D.; Awad, J.A.; Boss, H.J.; Blair, I.A.; Roberts, L.J., 2nd. Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. *Proceedings of the National Academy of Sciences of the United States of America* **1992**, *89*, 10721-10725, doi:10.1073/pnas.89.22.10721.
150. Roberts, L.J.; Morrow, J.D. Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. *Free radical biology & medicine* **2000**, *28*, 505-513, doi:10.1016/s0891-5849(99)00264-6.
151. Lawson, J.A.; Rokach, J.; FitzGerald, G.A. Isoprostanes: formation, analysis and use as indices of lipid peroxidation in vivo. *The Journal of biological chemistry* **1999**, *274*, 24441-24444, doi:10.1074/jbc.274.35.24441.

152. Banerjee, M.; Kang, K.H.; Morrow, J.D.; Roberts, L.J.; Newman, J.H. Effects of a novel prostaglandin, 8-epi-PGF2 alpha, in rabbit lung in situ. *The American journal of physiology* **1992**, *263*, H660-663, doi:10.1152/ajpheart.1992.263.3.H660.
153. Vacchiano, C.A.; Tempel, G.E. Role of nonenzymatically generated prostanoid, 8-iso-PGF2 alpha, in pulmonary oxygen toxicity. *Journal of Applied Physiology* **1994**, *77*, 2912-2917, doi:10.1152/jappl.1994.77.6.2912.
154. Wang, Z.; Ciabattoni, G.; Creminon, C.; Lawson, J.; Fitzgerald, G.A.; Patrono, C.; Maclouf, J. Immunological characterization of urinary 8-epi-prostaglandin F2 alpha excretion in man. *The Journal of pharmacology and experimental therapeutics* **1995**, *275*, 94-100.
155. Morrow, J.D.; Hill, K.E.; Burk, R.F.; Nammour, T.M.; Badr, K.F.; Roberts, L.J., 2nd. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc Natl Acad Sci U S A* **1990**, *87*, 9383-9387, doi:10.1073/pnas.87.23.9383.
156. Reilly, M.P.; Barry, P.; Lawson, J.A.; FitzGerald, G. Urinary 8-EPI PGF2α: an index of oxidant stress in vivo. *Fibrinolysis and Proteolysis* **1997**, *11*, 81-84, doi:https://doi.org/10.1016/S0268-9499(97)80029-X.
157. Pelclova, D.; Fenclova, Z.; Kacer, P.; Kuzma, M.; Navratil, T.; Lebedova, J. Increased 8-isoprostane, a marker of oxidative stress in exhaled breath condensate in subjects with asbestos exposure. *Industrial health* **2008**, *46*, 484-489, doi:10.2486/indhealth.46.484.
158. Jomova, K.; Valko, M. Advances in metal-induced oxidative stress and human disease. *Toxicology* **2011**, *283*, 65-87, doi:https://doi.org/10.1016/j.tox.2011.03.001.
159. Grimsrud, P.A.; Xie, H.; Griffin, T.J.; Bernlohr, D.A. Oxidative stress and covalent modification of protein with bioactive aldehydes. *The Journal of biological chemistry* **2008**, *283*, 21837-21841, doi:10.1074/jbc.R700019200.
160. Fritz, K.S.; Petersen, D.R. An overview of the chemistry and biology of reactive aldehydes. *Free radical biology & medicine* **2013**, *59*, 85-91, doi:10.1016/j.freeradbiomed.2012.06.025.
161. Slatter, D.A.; Murray, M.; Bailey, A.J. Formation of a dihydropyridine derivative as a potential cross-link derived from malondialdehyde in physiological systems. *FEBS Letters* **1998**, *421*, 180-184, doi:10.1016/S0014-5793(97)01554-8.
162. Steinberg, D.; Parthasarathy, S.; Carew, T.E.; Khoo, J.C.; Witztum, J.L. Beyond Cholesterol. *New England Journal of Medicine* **1989**, *320*, 915-924, doi:10.1056/NEJM198904063201407.
163. Palinski, W.; Ord, V.A.; Plump, A.S.; Breslow, J.L.; Steinberg, D.; Witztum, J.L. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. *Arteriosclerosis and thrombosis : a journal of vascular biology* **1994**, *14*, 605-616, doi:10.1161/01.atv.14.4.605.
164. Barrera, G.; Pizzimenti, S.; Daga, M.; Dianzani, C.; Arcaro, A.; Cetrangolo, G.P.; Giordano, G.; Cucci, M.A.; Graf, M.; Gentile, F. Lipid Peroxidation-Derived Aldehydes, 4-Hydroxynonenal and Malondialdehyde in Aging-Related Disorders. *Antioxidants (Basel)* **2018**, *7*, 102, doi:10.3390/antiox7080102.
165. Cui, X.; Gong, J.; Han, H.; He, L.; Teng, Y.; Tetley, T.; Sinharay, R.; Chung, K.F.; Islam, T.; Gilliland, F.; et al. Relationship between free and total malondialdehyde, a well-established marker of oxidative stress, in various types of human biospecimens. *2018* **2018**, *10*, 3088-3197.
166. Di Minno, A.; Turnu, L.; Porro, B.; Squellerio, I.; Cavalca, V.; Tremoli, E.; Di Minno, M.N.D. 8-Hydroxy-2-deoxyguanosine levels and heart failure: A systematic review and meta-analysis of the literature. *Nutrition, Metabolism and Cardiovascular Diseases* **2017**, *27*, 201-208, doi:https://doi.org/10.1016/j.numecd.2016.10.009.
167. Ames, B.N.; Gold, L.S. Endogenous mutagens and the causes of aging and cancer. *Mutation research* **1991**, *250*, 3-16, doi:10.1016/0027-5107(91)90157-j.
168. Halliwell, B. Effect of diet on cancer development: is oxidative DNA damage a biomarker? *Free radical biology & medicine* **2002**, *32*, 968-974, doi:10.1016/s0891-5849(02)00808-0.

169. Dizdaroglu, M. Chemical determination of free radical-induced damage to DNA. *Free radical biology & medicine* **1991**, *10*, 225-242, doi:10.1016/0891-5849(91)90080-m.
170. Cheng, K.C.; Cahill, D.S.; Kasai, H.; Nishimura, S.; Loeb, L.A. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. *The Journal of biological chemistry* **1992**, *267*, 166-172.
171. Kasai, H. Analysis of a form of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutation research* **1997**, *387*, 147-163, doi:10.1016/s1383-5742(97)00035-5.
172. Shigenaga, M.K.; Gimeno, C.J.; Ames, B.N. Urinary 8-hydroxy-2'-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. *Proc Natl Acad Sci U S A* **1989**, *86*, 9697-9701, doi:10.1073/pnas.86.24.9697.
173. Valavanidis, A.; Vlachogianni, T.; Fiotakis, C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis. *Journal of Environmental Science and Health, Part C* **2009**, *27*, 120-139, doi:10.1080/10590500902885684.
174. Andreoli, R.; Mutti, A.; Goldoni, M.; Manini, P.; Apostoli, P.; De Palma, G. Reference ranges of urinary biomarkers of oxidized guanine in (2'-deoxy)ribonucleotides and nucleic acids. *Free radical biology & medicine* **2011**, *50*, 254-261, doi:10.1016/j.freeradbiomed.2010.11.009.
175. Loft, S.; Poulsen, H.E. Markers of oxidative damage to DNA: antioxidants and molecular damage. *Methods in enzymology* **1999**, *300*, 166-184, doi:10.1016/s0076-6879(99)00124-x.
176. Scheideler, L.; Manke, H.-G.; Schwulera, U.; Inacker, O.; Hämmerle, H. Detection of Nonvolatile Macromolecules in Breath: A Possible Diagnostic Tool? *American Review of Respiratory Disease* **1993**, *148*, 778-784, doi:10.1164/ajrccm/148.3.778.
177. Gessner, C.; Kuhn, H.; Seyfarth, H.J.; Pankau, H.; Winkler, J.; Schauer, J.; Wirtz, H. Factors influencing breath condensate volume. *Pneumologie* **2001**, *55*, 414-419, doi:10.1055/s-2001-16947.
178. Horváth, I.; Barnes, P.J.; Loukides, S.; Sterk, P.J.; Högman, M.; Olin, A.-C.; Amann, A.; Antus, B.; Baraldi, E.; Bikov, A.; et al. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. *European Respiratory Journal* **2017**, *49*, 1600965, doi:10.1183/13993003.00965-2016.
179. Horváth, I.; Hunt, J.; Barnes, P.J.; Alving, K.; Antczak, A.; Baraldi, E.; Becher, G.; van Beurden, W.J.; Corradi, M.; Dekhuijzen, R.; et al. Exhaled breath condensate: methodological recommendations and unresolved questions. *Eur Respir J* **2005**, *26*, 523-548, doi:10.1183/09031936.05.00029705.
180. Horváth, I.; Hunt, J.; Barnes, P.J. Exhaled breath condensate: methodological recommendations and unresolved questions. *European Respiratory Journal* **2005**, *26*, 523, doi:10.1183/09031936.05.00029705.
181. Kharitonov, S.A.; Barnes, P.J. Biomarkers of some pulmonary diseases in exhaled breath. *Biomarkers* **2002**, *7*, 1-32, doi:10.1080/13547500110104233.
182. Jackson, A.S.; Sandrini, A.; Campbell, C.; Chow, S.; Thomas, P.S.; Yates, D.H. Comparison of Biomarkers in Exhaled Breath Condensate and Bronchoalveolar Lavage. *American journal of respiratory and critical care medicine* **2007**, *175*, 222-227, doi:10.1164/rccm.200601-107OC.
183. Syslová, K.; Kacer, P.; Kuzma, M.; Klusácková, P.; Fenclová, Z.; Lebedová, J.; Pelclová, D. Determination of 8-iso-prostaglandin F(2alpha) in exhaled breath condensate using combination of immunoseparation and LC-ESI-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* **2008**, *867*, 8-14, doi:10.1016/j.jchromb.2008.02.019.
184. Goldoni, M.; Corradi, M.; Mozzoni, P.; Folesani, G.; Alinovi, R.; Pinelli, S.; Andreoli, R.; Pigini, D.; Tillo, R.; Filetti, A.; et al. Concentration of exhaled breath condensate biomarkers after fractionated collection based on exhaled CO2 signal. *Journal of breath research* **2013**, *7*, 017101, doi:10.1088/1752-7155/7/1/017101.
185. Effros, R.M.; Hoagland, K.W.; Bosbous, M.; Castillo, D.; Foss, B.; Dunning, M.; Gare, M.; Lin, W.E.N.; Sun, F. Dilution of Respiratory Solutes in Exhaled Condensates. *American Journal of*

Respiratory and Critical Care Medicine **2002**, *165*, 663-669, doi:10.1164/ajrccm.165.5.2101018.

186. McCafferty, J.B.; Bradshaw, T.A.; Tate, S.; Greening, A.P.; Innes, J.A. Effects of breathing pattern and inspired air conditions on breath condensate volume, pH, nitrite, and protein concentrations. *Thorax* **2004**, *59*, 694-698, doi:10.1136/thx.2003.016949.
187. Schwarz, K.; Biller, H.; Windt, H.; Koch, W.; Hohlfeld, J.M. Characterization of exhaled particles from the healthy human lung--a systematic analysis in relation to pulmonary function variables. *J Aerosol Med Pulm Drug Deliv* **2010**, *23*, 371-379, doi:10.1089/jamp.2009.0809.
188. Fairchild, C.I.; Stampfer, J.F. Particle concentration in exhaled breath. *Am Ind Hyg Assoc J* **1987**, *48*, 948-949, doi:10.1080/15298668791385868.
189. Effros, R.M.; Dunning, M.B., 3rd; Biller, J.; Shaker, R. The promise and perils of exhaled breath condensates. *Am J Physiol Lung Cell Mol Physiol* **2004**, *287*, L1073-1080, doi:10.1152/ajplung.00069.2004.
190. Ashmawi, S.A.; Dewdar, I.; Mohamed, N.; Elhefny, A. Measurement of 8-isoprostane in exhaled breath condensate of patients with chronic obstructive pulmonary disease. *The Egyptian Journal of Chest Diseases and Tuberculosis* **2018**, *67*, 226, doi:10.4103/ejcdt.ejcdt_34_17.
191. Carpagnano, G.E.; Kharitonov, S.A.; Foschino-Barbaro, M.P.; Resta, O.; Gramiccioni, E.; Barnes, P.J. Supplementary oxygen in healthy subjects and those with COPD increases oxidative stress and airway inflammation. *Thorax* **2004**, *59*, 1016-1019, doi:10.1136/thx.2003.020768.
192. Kaźmierczak, M.; Ciebiada, M.; Pękala-Wojciechowska, A.; Pawłowski, M.; Pietras, T.; Antczak, A. Correlation of inflammatory markers with echocardiographic parameters of left and right ventricular function in patients with chronic obstructive pulmonary disease and cardiovascular diseases. *Pol Arch Med Wewn* **2014**, *124*, 290-297, doi:10.20452/pamw.2291.
193. Ko, F.W.; Lau, C.Y.; Leung, T.F.; Wong, G.W.; Lam, C.W.; Hui, D.S. Exhaled breath condensate levels of 8-isoprostane, growth related oncogene alpha and monocyte chemoattractant protein-1 in patients with chronic obstructive pulmonary disease. *Respir Med* **2006**, *100*, 630-638, doi:10.1016/j.rmed.2005.08.009.
194. Makris, D.; Paraskakis, E.; Korakas, P.; Karagiannakis, E.; Sourvinos, G.; Siafakas, N.M.; Tzanakis, N. Exhaled Breath Condensate 8-Isoprostane, Clinical Parameters, Radiological Indices and Airway Inflammation in COPD. *Respiration* **2008**, *75*, 138-144, doi:10.1159/000106377.
195. Antus, B.; Harnasi, G.; Drozdovszky, O.; Barta, I. Monitoring oxidative stress during chronic obstructive pulmonary disease exacerbations using malondialdehyde. *Respirology* **2014**, *19*, 74-79, doi:10.1111/resp.12155.
196. Antczak, A.; Piotrowski, W.; Marczak, J.; Ciebiada, M.; Gorski, P.; Barnes, P.J. Correlation between eicosanoids in bronchoalveolar lavage fluid and in exhaled breath condensate. *Dis Markers* **2011**, *30*, 213-220, doi:10.3233/dma-2011-0776.
197. Biernacki, W.A.; Kharitonov, S.A.; Barnes, P.J. Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax* **2003**, *58*, 294-298, doi:10.1136/thorax.58.4.294.
198. Brindicci, C.; Ito, K.; Torre, O.; Barnes, P.J.; Kharitonov, S.A. Effects of aminoguanidine, an inhibitor of inducible nitric oxide synthase, on nitric oxide production and its metabolites in healthy control subjects, healthy smokers, and COPD patients. *Chest* **2009**, *135*, 353-367, doi:10.1378/chest.08-0964.
199. Fritscher, L.G.; Post, M.; Rodrigues, M.T.; Silverman, F.; Balter, M.; Chapman, K.R.; Zamel, N. Profile of eicosanoids in breath condensate in asthma and COPD. *Journal of Breath Research* **2012**, *6*, 026001, doi:10.1088/1752-7155/6/2/026001.
200. Inonu, H.; Doruk, S.; Sahin, S.; Erkorkmaz, U.; Celik, D.; Celikel, S.; Seyfikli, Z. Oxidative Stress Levels in Exhaled Breath Condensate Associated With COPD and Smoking. *Respiratory Care* **2012**, *57*, 413, doi:10.4187/respcare.01302.
201. Kostikas, K.; Papatheodorou, G.; Ganas, K.; Psathakis, K.; Panagou, P.; Loukides, S. pH in expired breath condensate of patients with inflammatory airway diseases. *Am J Respir Crit Care Med* **2002**, *165*, 1364-1370, doi:10.1164/rccm.200111-068OC.

202. Montuschi, P.; Collins, J.V.; Ciabattoni, G.; Lazzeri, N.; Corradi, M.; Kharitonov, S.A.; Barnes, P.J. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med* **2000**, *162*, 1175-1177, doi:10.1164/ajrccm.162.3.2001063.
203. Pirozzi, C.; Sturrock, A.; Weng, H.-Y.; Greene, T.; Scholand, M.B.; Kanner, R.; Iii, R.P. Effect of Naturally Occurring Ozone Air Pollution Episodes on Pulmonary Oxidative Stress and Inflammation. *International Journal of Environmental Research and Public Health* **2015**, *12*, doi:10.3390/ijerph120505061.
204. Romero, P.V.; Rodríguez, B.; Martínez, S.; Cañizares, R.; Sepúlveda, D.; Manresa, F. Analysis of Oxidative Stress in Exhaled Breath Condensate From Patients With Severe Pulmonary Infections. *Archivos de Bronconeumología ((English Edition))* **2006**, *42*, 113-119, doi:https://doi.org/10.1016/S1579-2129(06)60128-6.
205. Santini, G.; Mores, N.; Shohreh, R.; Valente, S.; Dabrowska, M.; Trové, A.; Zini, G.; Cattani, P.; Fuso, L.; Mautone, A.; et al. Exhaled and non-exhaled non-invasive markers for assessment of respiratory inflammation in patients with stable COPD and healthy smokers. *J Breath Res* **2016**, *10*, 017102, doi:10.1088/1752-7155/10/1/017102.
206. Klein, E.; Adir, Y.; Krencel, A.; Peri, R.; Vasserman, B.; Fireman, E.; Kessel, A. Ultrafine particles in airways: A novel marker of COPD exacerbation risk and inflammatory status. *International Journal of Chronic Obstructive Pulmonary Disease* **2019**, *Volume 14*, 557-564, doi:10.2147/COPD.S187560.
207. Bartoli, M.L.; Novelli, F.; Costa, F.; Malagrinò, L.; Melosini, L.; Bacci, E.; Cianchetti, S.; Dente, F.L.; Di Franco, A.; Vagaggini, B.; et al. Malondialdehyde in Exhaled Breath Condensate as a Marker of Oxidative Stress in Different Pulmonary Diseases. *Mediators of Inflammation* **2011**, *2011*, 891752, doi:10.1155/2011/891752.
208. Corradi, M.; Rubinstein, I.; Andreoli, R.; Manini, P.; Caglieri, A.; Poli, D.; Alinovi, R.; Mutti, A. Aldehydes in exhaled breath condensate of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **2003**, *167*, 1380-1386, doi:10.1164/rccm.200210-1253OC.
209. Corradi, M.; Pignatti, P.; Manini, P.; Andreoli, R.; Goldoni, M.; Poppa, M.; Moscato, G.; Balbi, B.; Mutti, A. Comparison between exhaled and sputum oxidative stress biomarkers in chronic airway inflammation. *Eur Respir J* **2004**, *24*, 1011-1017, doi:10.1183/09031936.04.00002404.
210. Lee, J.S.; Shin, J.H.; Hwang, J.-H.; Baek, J.E.; Choi, B.-S. Malondialdehyde and 3-Nitrotyrosine in Exhaled Breath Condensate in Retired Elderly Coal Miners with Chronic Obstructive Pulmonary Disease. *Safety and Health at Work* **2014**, *5*, 91-96, doi:https://doi.org/10.1016/j.shaw.2014.03.001.
211. Maskey-Warzęchowska, M.; Nejman-Gryz, P.; Osinka, K.; Lis, P.; Malesa, K.; Górska, K.; Krenke, R. Acute Response to Cigarette Smoking Assessed in Exhaled Breath Condensate in Patients with Chronic Obstructive Pulmonary Disease and Healthy Smokers. In *Respiratory Treatment and Prevention*, Pokorski, M., Ed.; Springer International Publishing: Cham, 2017; pp. 73-80.
212. Dragonieri, S.; Quaranta, V.N.; Carratu, P.; Ranieri, T.; Resta, O. Exhaled breath profiling in patients with COPD and OSA overlap syndrome: a pilot study. *Journal of Breath Research* **2016**, *10*, 041001, doi:10.1088/1752-7155/10/4/041001.
213. Banga, I.; Paul, A.; Sardesai, A.U.; Muthukumar, S.; Prasad, S. ZEUS (ZIF-based electrochemical ultrasensitive screening) device for isopentane analytics with focus on lung cancer diagnosis. *RSC Advances* **2021**, *11*, 20519-20528, doi:10.1039/D1RA03093K.
214. Qiao, X.; Chen, X.; Huang, C.; Li, A.; Li, X.; Lu, Z.; Wang, T. Detection of Exhaled Volatile Organic Compounds Improved by Hollow Nanocages of Layered Double Hydroxide on Ag Nanowires. *Angewandte Chemie International Edition* **2019**, *58*, 16523-16527, doi:https://doi.org/10.1002/anie.201910865.
215. Goekce, S.; Concha-Lozano, N.; Sauvain, J.-J.; Hemmendinger, M.; Portela, A.; Sergent, E.; Andujar, P.; Pairon, J.-C.; Wild, P.; Suárez, G. Multiscattering-enhanced absorbance to enable the sensitive analysis of extremely diluted biological samples: Determination of oxidative

potential in exhaled air. *Medicine in Novel Technology and Devices* **2022**, *14*, 100120, doi:https://doi.org/10.1016/j.medntd.2022.100120.

216. Tsikas, D. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Anal Biochem* **2017**, *524*, 13-30, doi:10.1016/j.ab.2016.10.021.
217. Richardson, S.D.; Caughran, T.V.; Poiger, T.; Guo, Y.; Crumley, F.G. Application of DNPH Derivatization with LC/MS to the Identification of Polar Carbonyl Disinfection Byproducts in Drinking Water. *Ozone: Science & Engineering* **2000**, *22*, 653-675, doi:10.1080/01919510009408805.
218. Martinez, M.P.; Kannan, K. Simultaneous Analysis of Seven Biomarkers of Oxidative Damage to Lipids, Proteins, and DNA in Urine. *Environ Sci Technol* **2018**, *52*, 6647-6655, doi:10.1021/acs.est.8b00883.
219. Chen, J.-L.; Huang, Y.-J.; Pan, C.-H.; Hu, C.-W.; Chao, M.-R. Determination of urinary malondialdehyde by isotope dilution LC-MS/MS with automated solid-phase extraction: A cautionary note on derivatization optimization. *Free Radical Biology and Medicine* **2011**, *51*, 1823-1829, doi:https://doi.org/10.1016/j.freeradbiomed.2011.08.012.
220. Horváth, I.; Barnes, P.J.; Loukides, S.; Sterk, P.J.; Högman, M.; Olin, A.C.; Amann, A.; Antus, B.; Baraldi, E.; Bikov, A.; et al. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. *Eur Respir J* **2017**, *49*, doi:10.1183/13993003.00965-2016.
221. FDA, U.S. Bioanalytical Method Validation Guidance for Industry. *United States Food & Drug Administration* **2018**, 44.
222. Anderson, D.J. Determination of the lower limit of detection. *Clinical Chemistry* **1989**, *35*, 2152-2153, doi:10.1093/clinchem/35.10.2152.
223. Boulanger, B.; Chiap, P.; Dewe, W.; Crommen, J.; Hubert, P. An analysis of the SFSTP guide on validation of chromatographic bioanalytical methods: progress and limitations. *Journal of pharmaceutical and biomedical analysis* **2003**, *32*, 753-765, doi:10.1016/s0731-7085(03)00182-1.
224. Ellison SLR., W.A. Quantifying uncertainty in analytical measurement. *Eurachem-CITAC Guide CG4. https://www.eurachem.org/images/stories/Guides/pdf/QUAM2012_P1.pdf* **2012**.
225. Gu, H.; Liu, G.; Wang, J.; Aubry, A.F.; Arnold, M.E. Selecting the correct weighting factors for linear and quadratic calibration curves with least-squares regression algorithm in bioanalytical LC-MS/MS assays and impacts of using incorrect weighting factors on curve stability, data quality, and assay performance. *Anal Chem* **2014**, *86*, 8959-8966, doi:10.1021/ac5018265.
226. Hornung, R.W.; Reed, L.D. Estimation of Average Concentration in the Presence of Nondetectable Values. *Applied Occupational and Environmental Hygiene* **1990**, *5*, 46-51, doi:10.1080/1047322X.1990.10389587.
227. Chen, I.C.; Bertke, S.J.; Curwin, B.D. Quantile regression for exposure data with repeated measures in the presence of non-detects. *Journal of Exposure Science & Environmental Epidemiology* **2021**, doi:10.1038/s41370-021-00345-1.
228. Manini, P.; Andreoli, R.; Sforza, S.; Dall'Asta, C.; Galaverna, G.; Mutti, A.; Niessen, W.M.A. Evaluation of Alternate Isotope-Coded Derivatization Assay (AIDA) in the LC–MS/MS analysis of aldehydes in exhaled breath condensate. *Journal of Chromatography B* **2010**, *878*, 2616-2622, doi:https://doi.org/10.1016/j.jchromb.2010.02.010.
229. Pelclova, D.; Zdimal, V.; Kacer, P.; Zikova, N.; Komarc, M.; Fenclova, Z.; Vlckova, S.; Schwarz, J.; Makeš, O.; Syslova, K.; et al. Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO2 production workers. *Nanotoxicology* **2017**, *11*, 52-63, doi:10.1080/17435390.2016.1262921.
230. Casimirri, E.; Stendardo, M.; Bonci, M.; Andreoli, R.; Bottazzi, B.; Leone, R.; Schito, M.; Vaccari, A.; Papi, A.; Contoli, M.; et al. Biomarkers of oxidative-stress and inflammation in exhaled breath condensate from hospital cleaners. *Biomarkers* **2016**, *21*, 115-122, doi:10.3109/1354750X.2015.1118541.

231. Pelclova, D.; Zdimal, V.; Kacer, P.; Fenclova, Z.; Vlckova, S.; Syslova, K.; Navratil, T.; Schwarz, J.; Zikova, N.; Barosova, H.; et al. Oxidative stress markers are elevated in exhaled breath condensate of workers exposed to nanoparticles during iron oxide pigment production. *J Breath Res* **2016**, *10*, 016004, doi:10.1088/1752-7155/10/1/016004.
232. Mendonça, R.; Gning, O.; Di Cesaré, C.; Lachat, L.; Bennett, N.C.; Helfenstein, F.; Glauser, G. Sensitive and selective quantification of free and total malondialdehyde in plasma using UHPLC-HRMS. *Journal of lipid research* **2017**, *58*, 1924-1931, doi:10.1194/jlr.D076661.
233. Syslová, K.; Kacer, P.; Kuzma, M.; Najmanová, V.; Fenclová, Z.; Vlcková, S.; Lebedová, J.; Pelclová, D. Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **2009**, *877*, 2477-2486, doi:10.1016/j.jchromb.2009.06.008.
234. Beyer, H. Tukey, John W.: Exploratory Data Analysis. Addison-Wesley Publishing Company Reading, Mass. — Menlo Park, Cal., London, Amsterdam, Don Mills, Ontario, Sydney 1977, XVI, 688 S. *Biometrical Journal* **1981**, *23*, 413-414, doi:https://doi.org/10.1002/bimj.4710230408.
235. Carter, S.R.; Davis, C.S.; Kovacs, E.J. Exhaled breath condensate collection in the mechanically ventilated patient. *Respir Med* **2012**, *106*, 601-613, doi:10.1016/j.rmed.2012.02.003.
236. Milatovic, D.; Montine, T.J.; Aschner, M. Measurement of isoprostanes as markers of oxidative stress. *Methods Mol Biol* **2011**, *758*, 195-204, doi:10.1007/978-1-61779-170-3_13.
237. Syslová, K.; Kačer, P.; Kuzma, M.; Pankrácová, A.; Fenclová, Z.; Vlčková, Š.; Lebedová, J.; Pelclová, D. LC-ESI-MS/MS method for oxidative stress multimarker screening in the exhaled breath condensate of asbestosis/silicosis patients. *Journal of Breath Research* **2010**, *4*, 017104, doi:10.1088/1752-7155/4/1/017104.
238. Peel, A.M.; Crossman-Barnes, C.J.; Tang, J.; Fowler, S.J.; Davies, G.A.; Wilson, A.M.; Loke, Y.K. Biomarkers in adult asthma: a systematic review of 8-isoprostane in exhaled breath condensate. *J Breath Res* **2017**, *11*, 016011, doi:10.1088/1752-7163/aa5a8a.
239. Carpenter, C.T.; Price, P.V.; Christman, B.W. Exhaled breath condensate isoprostanes are elevated in patients with acute lung injury or ARDS. *Chest* **1998**, *114*, 1653-1659, doi:10.1378/chest.114.6.1653.
240. Cruickshank-Quinn, C.; Armstrong, M.; Powell, R.; Gomez, J.; Elie, M.; Reisdorph, N. Determining the presence of asthma-related molecules and salivary contamination in exhaled breath condensate. *Respir Res* **2017**, *18*, 57, doi:10.1186/s12931-017-0538-5.
241. Tufvesson, E.; Bjermer, L. Methodological improvements for measuring eicosanoids and cytokines in exhaled breath condensate. *Respir Med* **2006**, *100*, 34-38, doi:10.1016/j.rmed.2005.04.007.
242. Stamatakis, K.; PÉRez-Sala, D. Prostanoids with Cyclopentenone Structure as Tools for the Characterization of Electrophilic Lipid–Protein Interactomes. *Annals of the New York Academy of Sciences* **2006**, *1091*, 548-570, doi:https://doi.org/10.1196/annals.1378.096.
243. Wang, C.J.; Yang, N.H.; Liou, S.H.; Lee, H.L. Fast quantification of the exhaled breath condensate of oxidative stress 8-iso-prostaglandin F2alpha using on-line solid-phase extraction coupled with liquid chromatography/electrospray ionization mass spectrometry. *Talanta* **2010**, *82*, 1434-1438, doi:10.1016/j.talanta.2010.07.015.
244. Guseva Canu, I.; Hemmendinger, M.; Sauvain, J.J.; Suarez, G.; Hopf, N.B.; Pralong, J.A.; Ben Rayana, T.; Besançon, S.; Sakthithasan, K.; Jouannique, V.; et al. Respiratory Disease Occupational Biomonitoring Collaborative Project (ROBoCoP): A longitudinal pilot study and implementation research in the Parisian transport company. *J Occup Med Toxicol* **2021**, *16*, 22, doi:10.1186/s12995-021-00312-4.
245. Scheideler, L.; Manke, H.G.; Schwulera, U.; Inacker, O.; Hämmerle, H. Detection of nonvolatile macromolecules in breath. A possible diagnostic tool? *Am Rev Respir Dis* **1993**, *148*, 778-784, doi:10.1164/ajrccm/148.3.778.

246. Meng, X.; Liu, Q.; Ding, Y. Paper-based solid-phase microextraction for analysis of 8-hydroxy-2'-deoxyguanosine in urine sample by CE-LIF. *Electrophoresis* **2017**, *38*, 494-500, doi:10.1002/elps.201600439.
247. Suárez G, S.C., Martin OJF: Optical method and device for identifying analytes EP Patent PCT/IB2011/055819. **2011**.
248. Suárez, G.; Santschi, C.; Slaveykova, V.I.; Martin, O. Sensing the dynamics of oxidative stress using enhanced absorption in protein-loaded random media. *Scientific reports* **2013**, *3*, 3447, doi:10.1038/srep03447.
249. Suarez, G.; Santschi, C.; Plateel, G.; Martin, O.J.; Riediker, M. Absorbance enhancement in microplate wells for improved-sensitivity biosensors. *Biosensors & bioelectronics* **2014**, *56*, 198-203, doi:10.1016/j.bios.2013.12.063.
250. Miekisch, W.; Kischkel, S.; Sawacki, A.; Liebau, T.; Mieth, M.; Schubert, J.K. Impact of sampling procedures on the results of breath analysis. *Journal of breath research* **2008**, *2*, 026007, doi:10.1088/1752-7155/2/2/026007.
251. Strasser, G.; Hiebaum, S.; Neuberger, M. Commuter exposure to fine and ultrafine particulate matter in Vienna. *Wiener klinische Wochenschrift* **2018**, *130*, 62-69, doi:10.1007/s00508-017-1274-z.
252. Posselt, K.-P.; Neuberger, M.; Köhler, D. Fine and ultrafine particle exposure during commuting by subway in Vienna. *Wiener klinische Wochenschrift* **2019**, *131*, 374-380, doi:10.1007/s00508-019-1516-3.
253. Ozgen, S.; Ripamonti, G.; Malandrini, A.; Ragettli, M.S.; Lonati, G. Particle number and mass exposure concentrations by commuter transport modes in Milan, Italy. *AIMS Environmental Science* **2016**, *3*, 168-184, doi:doi:10.3934/environsci.2016.2.168 shu.
254. Yan, C.; Zheng, M.; Yang, Q.; Zhang, Q.; Qiu, X.; Zhang, Y.; Fu, H.; Li, X.; Zhu, T.; Zhu, Y. Commuter exposure to particulate matter and particle-bound PAHs in three transportation modes in Beijing, China. *Environmental Pollution* **2015**, *204*, 199-206, doi:https://doi.org/10.1016/j.envpol.2015.05.001.
255. Moreno, T.; Reche, C.; Rivas, I.; Cruz Minguillón, M.; Martins, V.; Vargas, C.; Buonanno, G.; Parga, J.; Pandolfi, M.; Brines, M.; et al. Urban air quality comparison for bus, tram, subway and pedestrian commutes in Barcelona. *Environmental research* **2015**, *142*, 495-510, doi:https://doi.org/10.1016/j.envres.2015.07.022.
256. Smith PG, M.R., Ross DA. *Field Trials of Health Interventions: A Toolbox.*, 3rd Edition ed.; Oxford University Press: London, 2015; p. 444.
257. Adeloye, D.; Chua, S.; Lee, C.; Basquill, C.; Papana, A.; Theodoratou, E.; Nair, H.; Gasevic, D.; Sridhar, D.; Campbell, H.; et al. Global and regional estimates of COPD prevalence: Systematic review and meta-analysis. *J Glob Health* **2015**, *5*, 020415, doi:10.7189/jogh.05-020415.
258. Suarez, G. *OxAirDirect –115-151 Rapport Final Dispositif de détection portatif pour la mesure directe de marqueurs du stress oxydant (ROS) dans l'air expiré*; IST: Lausanne, 2017.
259. Sauvain, J.J.; Suarez, G.; Edmé, J.L.; Bezerra, O.M.; Silveira, K.G.; Amaral, L.S.; Carneiro, A.P.; Chérot-Kornobis, N.; Sobaszek, A.; Hulo, S. Method validation of nanoparticle tracking analysis to measure pulmonary nanoparticle content: the size distribution in exhaled breath condensate depends on occupational exposure. *J Breath Res* **2017**, *11*, 016010, doi:10.1088/1752-7163/aa56dd.
260. Sauvain, J.J.; Edmé, J.L.; Wild, P.; Suarez, G.; Bezerra, O.; Talvani, A.; Algranti, E.; Carneiro, A.P.S.; Chérot-Kornobis, N.; Sobaszek, A.; et al. Does exposure to inflammatory particles modify the pattern of anion in exhaled breath condensate? *J Breath Res* **2020**, *14*, 026005, doi:10.1088/1752-7163/ab5d88.
261. Jaffe, M. Ueber den Niederschlag welchen Pikrinsäure in normalen Harn erzeugt and über eine neue Reaction des Kreatinins. *Zeitschrift für Physiologische Chemie* **1986**, *10*, 391-400.
262. GOLD. Global Initiative for Chronic Obstructive Lung Disease. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. . Available online:

http://goldcopd.org/global-strategy-diagnosis-management-prevention-copd-2017/ (accessed on Mai 5, 2017).

263. Campagna, D.; Ihaddadene, K.; Randon, A.; Mattei, N.; Marchand, J.L.; Le Naour, C.; Imbernon, E. *Analyse de la mortalité des agents et ex-agents de la RATP sur la période 1980-1999 Cohorte EDGAR*; InVS-RATP: Paris, 2008; p. 24.
264. Quanjer, P.H.; Stanojevic, S.; Cole, T.J.; Baur, X.; Hall, G.L.; Culver, B.H.; Enright, P.L.; Hankinson, J.L.; Ip, M.S.; Zheng, J.; et al. ERS Global Lung Function Initiative. *The European respiratory journal* **2012**, *40*, 1324-1343, doi:doi: 10.1183/09031936.00080312.
265. Guseva Canu, I.; Crézé, C.; Hemmendinger, M.; Ben Rayana, T.; Besançon, S.; Jouannique, V.; Debatisse, A.; Wild, P.; Sauvain, J.J.; Suárez, G.; et al. Particle and metal exposure in Parisian subway: Relationship between exposure biomarkers in air, exhaled breath condensate, and urine. *Int J Hyg Environ Health* **2021**, *237*, 113837, doi:10.1016/j.ijheh.2021.113837.
266. Corradi, M.; Acampa, O.; Goldoni, M.; Adami, E.; Apostoli, P.; de Palma, G.; Pesci, A.; Mutti, A. Metallic elements in exhaled breath condensate of patients with interstitial lung diseases. *Journal of Breath Research* **2009**, *3*, 046003, doi:10.1088/1752-7155/3/4/046003.
267. Mutti, A.; Corradi, M.; Goldoni, M.; Vettori, M.V.; Bernard, A.; Apostoli, P. Exhaled metallic elements and serum pneumoproteins in asymptomatic smokers and patients with COPD or asthma. *Chest* **2006**, *129*, 1288-1297, doi:10.1378/chest.129.5.1288.
268. Ghio, A.J.; Madden, M.C.; Esther, C.R. Transition and post-transition metals in exhaled breath condensate. *Journal of breath research* **2018**, *12*, 027112-027112, doi:10.1088/1752-7163/aaa214.
269. Shoman, Y.; Wild, P.; Hemmendinger, M.; Graille, M.; Sauvain, J.J.; Hopf, N.B.; Guseva Canu, I. Reference Ranges of 8-Isoprostane Concentrations in Exhaled Breath Condensate (EBC): A Systematic Review and Meta-Analysis. *Int J Mol Sci* **2020**, *21*, doi:10.3390/ijms21113822.
270. Hemmendinger, M.; Wild, P.; Shoman, Y.; Graille, M.; Bergamaschi, E.; Hopf, N.; Guseva Canu, I. Reference ranges of oxidative stress biomarkers selected for non-invasive biological surveillance of nanotechnology workers: Study protocol and meta-analysis results for 8-OHdG in exhaled breath condensate. *Toxicol Lett* **2020**, *327*, 41-47, doi:10.1016/j.toxlet.2020.03.021.
271. Greguš, M.; Foret, F.; Kindlová, D.; Pokojová, E.; Plutinský, M.; Doubková, M.; Merta, Z.; Binková, I.; Skřičková, J.; Kubáň, P. Monitoring the ionic content of exhaled breath condensate in various respiratory diseases by capillary electrophoresis with contactless conductivity detection. *Journal of Breath Research* **2015**, *9*, 027107, doi:10.1088/1752-7155/9/2/027107.
272. Nisse, C.; Tagne-Fotso, R.; Howsam, M.; Richeval, C.; Labat, L.; Leroyer, A. Blood and urinary levels of metals and metalloids in the general adult population of Northern France: The IMEPOGE study, 2008-2010. *Int J Hyg Environ Health* **2017**, *220*, 341-363, doi:10.1016/j.ijheh.2016.09.020.
273. Morton, J.; Tan, E.; Leese, E.; Cocker, J. Determination of 61 elements in urine samples collected from a non-occupationally exposed UK adult population. *Toxicology Letters* **2014**, *231*, 179-193, doi:https://doi.org/10.1016/j.toxlet.2014.08.019.
274. Hoet, P.; Jacquerye, C.; Deumer, G.; Lison, D.; Haufroid, V. Reference values and upper reference limits for 26 trace elements in the urine of adults living in Belgium. *Clin Chem Lab Med* **2013**, *51*, 839-849, doi:10.1515/cclm-2012-0688.
275. Fréry, N.; Guldner, L.; A., S.; Garnier, R.; Zeghnoun, A.; Bidondo, M.L. *Exposition de la population française aux substances chimiques de l'environnement.* ; 2011; p. 151.
276. Graille, M.; Wild, P.; Sauvain, J.J.; Hemmendinger, M.; Guseva Canu, I.; Hopf, N.B. Urinary 8-OHdG as a Biomarker for Oxidative Stress: A Systematic Literature Review and Meta-Analysis. *Int J Mol Sci* **2020**, *21*, doi:10.3390/ijms21113743.
277. Graille, M.; Wild, P.; Sauvain, J.J.; Hemmendinger, M.; Guseva Canu, I.; Hopf, N.B. Urinary 8-isoprostane as a biomarker for oxidative stress. A systematic review and meta-analysis. *Toxicol Lett* **2020**, *328*, 19-27, doi:10.1016/j.toxlet.2020.04.006.
278. Ozarda, Y. Reference intervals: current status, recent developments and future considerations. *Biochem Med (Zagreb)* **2016**, *26*, 5-16, doi:10.11613/bm.2016.001.

279. Ozarda, Y.; Sikaris, K.; Streichert, T.; Macri, J. Distinguishing reference intervals and clinical decision limits - A review by the IFCC Committee on Reference Intervals and Decision Limits. *Crit Rev Clin Lab Sci* **2018**, *55*, 420-431, doi:10.1080/10408363.2018.1482256.
280. CLSI.; IFCC. *Defining, establishing and verifying reference intervals in the clinical laboratory: approved guideline*

Clinical and Laboratory Standards Institute and International Federation of Clinical Chemistry 2008; pp. 1-76.
281. Ouzzani, M.; Hammady, H.; Fedorowicz, Z.; Elmagarmid, A. Rayyan-a web and mobile app for systematic reviews. *Systematic reviews* **2016**, *5*, 210-210, doi:10.1186/s13643-016-0384-4.
282. Guyatt, G.H.; Oxman, A.D.; Vist, G.E.; Kunz, R.; Falck-Ytter, Y.; Alonso-Coello, P.; Schunemann, H.J. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. *BMJ (Clinical research ed.)* **2008**, *336*, 924-926, doi:10.1136/bmj.39489.470347.AD.
283. Graille, M.; Wild, P.; Sauvain, J.J.; Hemmendinger, M.; Guseva Canu, I.; Hopf, N.B. Concentrations of urinary 8-isoprostane, an oxidative stress biomarker, in the adult population: A systematic literature review and meta-analysis **(submitted)**.
284. Deeks, J.J.; Higgins, J.P.T.; Altman, D.G. Analysing data and undertaking meta-analyses. In Cochrane handbook for systematic reviews of interventions *Version 5.2.0. ed JPT Higgins and S Green, chp 9. Londond : the cochrane collaboration* **2017**.
285. Fireman Klein, E.; Adir, Y.; Krencel, A.; Peri, R.; Vasserman, B.; Fireman, E.; Kessel, A. Ultrafine particles in airways: a novel marker of COPD exacerbation risk and inflammatory status. *Int J Chron Obstruct Pulmon Dis* **2019**, *14*, 557-564, doi:10.2147/COPD.S187560.
286. Pelclova, D.; Zdimal, V.; Kacer, P.; Vlckova, S.; Fenclova, Z.; Navratil, T.; Komarc, M.; Schwarz, J.; Zikova, N.; Makes, O.; et al. Markers of nucleic acids and proteins oxidation among office workers exposed to air pollutants including (nano)TiO2 particles. *Neuro endocrinology letters* **2016**, *37*, 13-16.
287. Alfaro, M.F.; Walby, W.F.; Adams, W.C.; Schelegle, E.S. Breath condensate levels of 8-isoprostane and leukotriene B4 after ozone inhalation are greater in sensitive versus nonsensitive subjects. *Exp Lung Res* **2007**, *33*, 115-133, doi:10.1080/01902140701364367.
288. Alvarez, R.F.; Cuadrado, G.R.; Arias, R.A.; Hernandez, J.A.C.; Antequera, B.P.; Urrutia, M.I.; Clara, P.C. Snoring as a Determinant Factor of Oxidative Stress in the Airway of Patients with Obstructive Sleep Apnea. *Lung* **2016**, *194*, 469-473, doi:10.1007/s00408-016-9869-0.
289. Andrianjafimasy, M.; Zerimech, F.; Akiki, Z.; Huyvaert, H.; Le Moual, N.; Siroux, V.; Matran, R.; Dumas, O.; Nadif, R. Oxidative stress biomarkers and asthma characteristics in adults of the EGEA study. *Eur Respir J* **2017**, *50*, doi:10.1183/13993003.01193-2017.
290. Antczak, A.; Ciebiada, M.; Pietras, T.; Piotrowski, W.J.; Kurmanowska, Z.; Gorski, P. Exhaled eicosanoids and biomarkers of oxidative stress in exacerbation of chronic obstructive pulmonary disease. *Arch Med Sci* **2012**, *8*, 277-285, doi:10.5114/aoms.2012.28555.
291. Antczak, A.; Montuschi, P.; Kharitonov, S.; Gorski, P.; Barnes, P.J. Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma. *Am J Respir Crit Care Med* **2002**, *166*, 301-306, doi:10.1164/rccm.2101021.
292. Antczak, A.; Piotrowski, W.; Marczak, J.; Ciebiada, M.; Gorski, P.; Barnes, P.J. Correlation between eicosanoids in bronchoalveolar lavage fluid and in exhaled breath condensate. *Dis Markers* **2011**, *30*, 213-220, doi:10.1155/2011/323794.
293. Antonopoulou, S.; Loukides, S.; Papatheodorou, G.; Roussos, C.; Alchanatis, M. Airway inflammation in obstructive sleep apnea: is leptin the missing link? *Respir Med* **2008**, *102*, 1399-1405, doi:10.1016/j.rmed.2008.04.021.
294. Ashmawi, S.S.A.; Dewdar, I.A.; Mohamed, N.A.; Elhefny, A.A. Measurement of 8-isoprostane in exhaled breath condensate of patients with chronic obstructive pulmonary disease. *Egypt J Chest Dis Tu* **2018**, *67*, 226-230, doi:10.4103/ejcdt.ejcdt_34_17.

295. Barreto, M.; Villa, M.P.; Corradi, M.; Barberi, S.; Monaco, G.; Martella, S.; Bohmerova, Z.; Sabatino, G.; Ronchetti, R. Non-invasive assessment of airway inflammation in ship-engine workers. *Int J Immunopathol Pharmacol* **2006**, *19*, 601-608, doi:10.1177/039463200601900316.
296. Bastug, E.; Tasliyurt, T.; Kutluturk, F.; Sahin, S.; Yilmaz, A.; Sivgin, H.; Yelken, B.M.; Ozturk, B.; Yilmaz, A.; Sahin, S. Evaluation of oxidative status with exhaled breath 8-isoprostane levels in patients with hyperthyroidism. *Endocr Metab Immune Disord Drug Targets* **2013**, *13*, 306-310, doi:10.2174/18715303113136660043.
297. Borrill, Z.L.; Roy, K.; Vessey, R.S.; Woodcock, A.A.; Singh, D. Non-invasive biomarkers and pulmonary function in smokers. *Int J Chron Obstruct Pulmon Dis* **2008**, *3*, 171-183, doi:10.2147/copd.s1850.
298. Brussino, L.; Badiu, I.; Sciascia, S.; Bugiani, M.; Heffler, E.; Guida, G.; Malinovschi, A.; Bucca, C.; Rolla, G. Oxidative stress and airway inflammation after allergen challenge evaluated by exhaled breath condensate analysis. *Clin Exp Allergy* **2010**, *40*, 1642-1647, doi:10.1111/j.1365-2222.2010.03604.x.
299. Carpagnano, G.E.; Kharitonov, S.A.; Foschino-Barbaro, M.P.; Resta, O.; Gramiccioni, E.; Barnes, P.J. Supplementary oxygen in healthy subjects and those with COPD increases oxidative stress and airway inflammation. *Thorax* **2004**, *59*, 1016-1019, doi:10.1136/thx.2003.020768.
300. Carpagnano, G.E.; Kharitonov, S.A.; Resta, O.; Foschino-Barbaro, M.P.; Gramiccioni, E.; Barnes, P.J. Increased 8-isoprostane and interleukin-6 in breath condensate of obstructive sleep apnea patients. *Chest* **2002**, *122*, 1162-1167, doi:10.1378/chest.122.4.1162.
301. Chan, H.P.; Tran, V.; Lewis, C.; Thomas, P.S. Elevated levels of oxidative stress markers in exhaled breath condensate. *J Thorac Oncol* **2009**, *4*, 172-178, doi:10.1097/JTO.0b013e3181949eb9.
302. Chow, S.; Campbell, C.; Sandrini, A.; Thomas, P.S.; Johnson, A.R.; Yates, D.H. Exhaled breath condensate biomarkers in asbestos-related lung disorders. *Resp Med* **2009**, *103*, 1091-1097, doi:10.1016/j.rmed.2009.04.009.
303. Chow, S.; Thomas, P.S.; Malouf, M.; Yates, D.H. Exhaled breath condensate (EBC) biomarkers in pulmonary fibrosis. *J Breath Res* **2012**, *6*, 016004, doi:10.1088/1752-7155/6/1/016004.
304. Ciebiada, M.; Gorski, P.; Antczak, A. Eicosanoids in exhaled breath condensate and bronchoalveolar lavage fluid of patients with primary lung cancer. *Dis Markers* **2012**, *32*, 329-335, doi:10.3233/DMA-2011-0890.
305. Cruz, M.J.; Sanchez-Vidaurre, S.; Romero, P.V.; Morell, F.; Munoz, X. Impact Of Age on pH, 8-Isoprostane, and Nitrogen Oxides in Exhaled Breath Condensate. *Chest* **2009**, *135*, 462-467, doi:10.1378/chest.08-1007.
306. Do, R.; Bartlett, K.H.; Dimich-Ward, H.; Chu, W.; Kennedy, S.M. Biomarkers of airway acidity and oxidative stress in exhaled breath condensate from grain workers. *Am J Respir Crit Care Med* **2008**, *178*, 1048-1054, doi:10.1164/rccm.200711-1731OC.
307. Emilsson, O.I.; Benediktsdottir, B.; Olafsson, I.; Cook, E.; Juliusson, S.; Bjornsson, E.S.; Guethlaugsdottir, S.; Guethmundsdottir, A.S.; Mirgorodskaya, E.; Ljungstrom, E.; et al. Respiratory symptoms, sleep-disordered breathing and biomarkers in nocturnal gastroesophageal reflux. *Respir Res* **2016**, *17*, 115, doi:10.1186/s12931-016-0431-7.
308. Font-Ribera, L.; Kogevinas, M.; Zock, J.P.; Gomez, F.P.; Barreiro, E.; Nieuwenhuijsen, M.J.; Fernandez, P.; Lourencetti, C.; Perez-Olabarria, M.; Bustamante, M.; et al. Short-Term Changes in Respiratory Biomarkers after Swimming in a Chlorinated Pool. *Environ Health Persp* **2010**, *118*, 1538-1544, doi:10.1289/ehp.1001961.
309. Goldoni, M.; Corradi, M.; Mozzoni, P.; Folesani, G.; Alinovi, R.; Pinelli, S.; Andreoli, R.; Pigini, D.; Tillo, R.; Filetti, A.; et al. Concentration of exhaled breath condensate biomarkers after fractionated collection based on exhaled CO2 signal. *J Breath Res* **2013**, *7*, doi:Artn 017101

10.1088/1752-7155/7/1/017101.

310. Gratziou, C.; Rovina, N.; Makris, M.; Simoes, D.C.M.; Papapetropoulos, A.; Roussos, C. Breath Markers of Oxidative Stress and Airway Inflammation in Seasonal Allergic Rhinitis. *Int J Immunopath Ph* **2008**, *21*, 949-957, doi:Doi 10.1177/039463200802100419.
311. Hakim, F.; Hellou, E.; Goldbart, A.; Katz, R.; Bentur, Y.; Bentur, L. The Acute Effects of Water-Pipe Smoking on the Cardiorespiratory System. *Chest* **2011**, *139*, 775-781, doi:10.1378/chest.10-1833.
312. Heinicke, I.; Boehler, A.; Rechsteiner, T.; Bogdanova, A.; Jelkmann, W.; Hofer, M.; Rawlings, P.; Araneda, O.F.; Behn, C.; Gassmann, M.; et al. Moderate altitude but not additional endurance training increases markers of oxidative stress in exhaled breath condensate. *Eur J Appl Physiol* **2009**, *106*, 599-604, doi:10.1007/s00421-009-1014-9.
313. Hoffmeyer, F.; Raulf-Heimsoth, M.; Harth, V.; Bunger, J.; Bruning, T. Comparative analysis of selected exhaled breath biomarkers obtained with two different temperature-controlled devices. *BMC Pulm Med* **2009**, *9*, 48, doi:10.1186/1471-2466-9-48.
314. Hoffmeyer, F.; Raulf-Heimsoth, M.; Lehnert, M.; Kendzia, B.; Bernard, S.; Berresheim, H.; Duser, M.; Henry, J.; Weiss, T.; Koch, H.M.; et al. Impact of Different Welding Techniques on Biological Effect Markers in Exhaled Breath Condensate of 58 Mild Steel Welders. *J Toxicol Env Heal A* **2012**, *75*, 525-532, doi:10.1080/15287394.2012.675303.
315. Inonu, H.; Doruk, S.; Sahin, S.; Erkorkmaz, U.; Celik, D.; Celikel, S.; Seyfikli, Z. Oxidative stress levels in exhaled breath condensate associated with COPD and smoking. *Respir Care* **2012**, *57*, 413-419, doi:10.4187/respcare.01302.
316. Koskela, H.O.; Purokivi, M.K. Airway oxidative stress in chronic cough. *Cough* **2013**, *9*, 26, doi:10.1186/1745-9974-9-26.
317. Koskela, H.O.; Purokivi, M.K.; Nieminen, R.M.; Moilanen, E. Asthmatic cough and airway oxidative stress. *Resp Physiol Neurobi* **2012**, *181*, 346-350, doi:10.1016/j.resp.2012.04.006.
318. Koskela, K.; Oksa, P.; Sauni, R.; Linnainmaa, M.; Toivio, P.; Lehtimaki, L.; Moilanen, E.; Nieminen, R.; Luukkonen, R.; Uitti, J. Pulmonary inflammation in foundry workers. *J Occup Environ Med* **2015**, *57*, 124-128, doi:10.1097/JOM.0000000000000390.
319. Kurti, S.P.; Emerson, S.R.; Rosenkranz, S.K.; Teeman, C.S.; Emerson, E.M.; Cull, B.J.; Smith, J.R.; Harms, C.A. Post-prandial systemic 8-isoprostane increases after consumption of moderate and high-fat meals in insufficiently active males. *Nutr Res* **2017**, *39*, 61-68, doi:10.1016/j.nutres.2017.02.003.
320. Kurti, S.P.; Emerson, S.R.; Smith, J.R.; Rosenkranz, S.K.; Alexander, S.A.; Lovoy, G.M.; Harms, C.A. Older women exhibit greater airway 8-isoprostane responses to strenuous exercise compared with older men and younger controls. *Appl Physiol Nutr Metab* **2018**, *43*, 497-503, doi:10.1139/apnm-2017-0565.
321. Lehtimaki, L.; Oksa, P.; Jarvenpaa, R.; Vierikko, T.; Nieminen, R.; Kankaanranta, H.; Uitti, J.; Moilanen, E. Pulmonary inflammation in asbestos-exposed subjects with borderline parenchymal changes on HRCT. *Resp Med* **2010**, *104*, 1042-1049, doi:10.1016/j.rmed.2010.01.019.
322. Lehtonen, H.; Oksa, P.; Lehtimaki, L.; Sepponen, A.; Nieminen, R.; Kankaanranta, H.; Saarelainen, S.; Jarvenpaa, R.; Uitti, J.; Moilanen, E. Increased alveolar nitric oxide concentration and high levels of leukotriene B-4 and 8-isoprostane in exhaled breath condensate in patients with asbestosis. *Thorax* **2007**, *62*, 602-607, doi:10.1136/thx.2006.067868.
323. Leung, T.F.; Li, C.Y.; Yung, E.; Liu, E.K.; Lam, C.W.; Wong, G.W. Clinical and technical factors affecting pH and other biomarkers in exhaled breath condensate. *Pediatr Pulmonol* **2006**, *41*, 87-94, doi:10.1002/ppul.20296.
324. Li, Y.X.; Chongsuvivatwong, V.; Geater, A.; Liu, A. Exhaled breath condensate cytokine level as a diagnostic tool for obstructive sleep apnea syndrome. *Sleep Med* **2009**, *10*, 95-103, doi:10.1016/j.sleep.2007.11.013.

325. Liou, S.H.; Wu, W.T.; Liao, H.Y.; Chen, C.Y.; Tsai, C.Y.; Jung, W.T.; Lee, H.L. Global DNA methylation and oxidative stress biomarkers in workers exposed to metal oxide nanoparticles. *J Hazard Mater* **2017**, *331*, 329-335, doi:10.1016/j.jhazmat.2017.02.042.
326. Marie-Desvergne, C.; Dubosson, M.; Mossuz, V.C. Evaluation of a new method for the collection and measurement of 8-isoprostane in exhaled breath for future application in nanoparticle exposure biomonitoring. *J Breath Res* **2018**, *12*, doi:ARTN 031001

10.1088/1752-7163/aabdf2.
327. Mazur, W.; Stark, H.; Sovijarvi, A.; Myllarniemi, M.; Kinnula, V.L. Comparison of 8-Isoprostane and Interleukin-8 in Induced Sputum and Exhaled Breath Condensate from Asymptomatic and Symptomatic Smokers. *Respiration* **2009**, *78*, 209-216, doi:10.1159/000206010.
328. Montuschi, P.; Corradi, M.; Ciabattoni, G.; Nightingale, J.; Kharitonov, S.A.; Barnes, P.J. Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am J Respir Crit Care Med* **1999**, *160*, 216-220, doi:10.1164/ajrccm.160.1.9809140.
329. Montuschi, P.; Nightingale, J.A.; Kharitonov, S.A.; Barnes, P.J. Ozone-induced increase in exhaled 8-isoprostane in healthy subjects is resistant to inhaled budesonide. *Free Radical Bio Med* **2002**, *33*, 1403-1408, doi:Pii S0891-5849(02)01084-5

Doi 10.1016/S0891-5849(02)01084-5.
330. Papaioannou, A.I.; Koutsokera, A.; Tanou, K.; Kiropoulos, T.S.; Tsilioni, I.; Oikonomidi, S.; Liadaki, K.; Pournaras, S.; Gourgoulianis, K.I.; Kostikas, K. The acute effect of smoking in healthy and asthmatic smokers. *European Journal of Clinical Investigation* **2010**, *40*, 103-109, doi:10.1111/j.1365-2362.2009.02221.x.
331. Pekala-Wojciechowska, A.; Poznanski, M.; Szyszow, K.; Antczak, A. Concentration of 8-isoprostanes in the exhaled breath condensate as a marker of oxidative stress in patients with type 1 diabetes. *Adv Respir Med* **2018**, *86*, 3-6, doi:10.5603/Arm.2018.0002.
332. Pelclova, D.; Fenclova, Z.; Kacer, P.; Kuzma, M.; Navratil, T.; Lebedova, J. Increased 8-isoprostane, a marker of oxidative stress in exhaled breath condensate in subjects with asbestos exposure. *Ind Health* **2008**, *46*, 484-489, doi:DOI 10.2486/indhealth.46.484.
333. Pelclova, D.; Fenclova, Z.; Kacer, P.; Navratil, T.; Kuzma, M.; Lebedova, J.; Klusackova, P. 8-isoprostane and leukotrienes in exhaled breath condensate in Czech subjects with silicosis. *Ind Health* **2007**, *45*, 766-774, doi:DOI 10.2486/indhealth.45.766.
334. Pelclova, D.; Fenclova, Z.; Navratil, T.; Vlckova, S.; Syslova, K.; Kuzma, M.; Zdimal, V.; Schwarz, J.; Pusman, J.; Zikova, N.; et al. Markers of oxidative stress in exhaled breath condensate are significantly increased in workers exposed to aerosol containing TiO2 nanoparticles. *Toxicology Letters* **2014**, *229*, S12-S12, doi:10.1016/j.toxlet.2014.06.071.
335. Pelclova, D.; Fenclova, Z.; Syslova, K.; Vlckova, S.; Lebedova, J.; Pecha, O.; Belacek, J.; Navratil, T.; Kuzma, M.; Kacer, P. Oxidative Stress Markers in Exhaled Breath Condensate in Lung Fibroses Are Not Significantly Affected by Systemic Diseases. *Ind Health* **2011**, *49*, 746-754, doi:DOI 10.2486/indhealth.MS1237.
336. Pelclova, D.; Fenclova, Z.; Vlckova, S.; Klusackova, P.; Lebedova, J.; Syslova, K.; Belacek, J.; Kuzma, M.; Navratil, T.; Zakharov, S.; et al. Occupational asthma follow-up--which markers are elevated in exhaled breath condensate and plasma? *Int J Occup Med Environ Health* **2014**, *27*, 206-215, doi:10.2478/s13382-014-0243-2.
337. Pelclova, D.; Navratil, T.; Vlckova, S.; Fenclova, Z.; Pelcl, T.; Kacerova, T.; Kacer, P. Exhaled breath condensate biomarkers reflect systemic changes in patients with chronic dioxin intoxication. *Monatsh Chem* **2018**, *149*, 1579-1586, doi:10.1007/s00706-018-2211-1.
338. Pelclova, D.; Zdimal, V.; Kacer, P.; Fenclova, Z.; Vlckova, S.; Syslova, K.; Navratil, T.; Schwarz, J.; Zikova, N.; Barosova, H.; et al. Oxidative stress markers are elevated in exhaled breath condensate of workers exposed to nanoparticles during iron oxide pigment production. *J Breath Res* **2016**, *10*, doi:Artn 016004

10.1088/1752-7155/10/1/016004.

339. Pelclova, D.; Zdimal, V.; Kacer, P.; Komarc, M.; Fenclova, Z.; Vlckova, S.; Zikova, N.; Schwarz, J.; Makes, O.; Navratil, T.; et al. Markers of lipid oxidative damage among office workers exposed intermittently to air pollutants including nanoTiO(2) particles. *Rev Environ Health* **2017**, *32*, 193-200, doi:10.1515/reveh-2016-0030.
340. Pelclova, D.; Zdimal, V.; Kacer, P.; Zikova, N.; Komarc, M.; Fenclova, Z.; Vlckova, S.; Schwarz, J.; Makes, O.; Syslova, K.; et al. Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO2 production workers. *Nanotoxicology* **2017**, *11*, 52-63, doi:10.1080/17435390.2016.1262921.
341. Piotrowski, W.J.; Antczak, A.; Marczak, J.; Nawrocka, A.; Kurmanowska, Z.; Gorski, P. Eicosanoids in exhaled breath condensate and BAL fluid of patients with sarcoidosis. *Chest* **2007**, *132*, 589-596, doi:10.1378/chest.07-0215.
342. Piotrowski, W.J.; Kurmanowska, Z.; Antczak, A.; Marczak, J.; Ciebiada, M.; Gorski, P. Exhaled 8-isoprostane in sarcoidosis: relation to superoxide anion production by bronchoalveolar lavage cells. *Inflamm Res* **2010**, *59*, 1027-1032, doi:10.1007/s00011-010-0222-4.
343. Piotrowski, W.J.; Kurmanowska, Z.; Antczak, A.; Marczak, J.; Gorski, P. Exhaled 8-isoprostane as a prognostic marker in sarcoidosis. A short term follow-up. *Bmc Pulmonary Medicine* **2010**, *10*, doi:Artn 23

10.1186/1471-2466-10-23.
344. Piotrowski, W.J.; Majewski, S.; Marczak, J.; Kurmanowska, Z.; Gorski, P.; Antczak, A. Exhaled breath 8-isoprostane as a marker of asthma severity. *Archives of Medical Science* **2012**, *8*, 515-520, doi:10.5114/aoms.2012.28639.
345. Pirozzi, C.; Sturrock, A.; Carey, P.; Whipple, S.; H., H.; Baker, J.; Weng, H.; Greene, M.; Scholand, M.; Kanner, R.; et al. Respiratory effects of particulate air pollution episodes in former smokers with and without chronic obstructive pulmonary disease: a panel study. *COPD Research and Practice* **2015**, doi:https://doi.org/10.1186/s40749-015-0004-z.
346. Pirozzi, C.; Sturrock, A.; Weng, H.Y.; Greene, T.; Scholand, M.B.; Kanner, R.; Paine, R., 3rd. Effect of naturally occurring ozone air pollution episodes on pulmonary oxidative stress and inflammation. *Int J Environ Res Public Health* **2015**, *12*, 5061-5075, doi:10.3390/ijerph120505061.
347. Psathakis, K.; Mermigkis, D.; Papatheodorou, G.; Loukides, S.; Panagou, P.; Polychronopoulos, V.; Siafakas, N.M.; Bouros, D. Exhaled markers of oxidative stress in idiopathic pulmonary fibrosis. *Eur J Clin Invest* **2006**, *36*, 362-367, doi:10.1111/j.1365-2362.2006.01636.x.
348. Psathakis, K.; Papatheodorou, G.; Plataki, M.; Panagou, P.; Loukides, S.; Siafakas, N.M.; Bouros, D. 8-Isoprostane, a marker of oxidative stress, is increased in the expired breath condensate of patients with pulmonary sarcoidosis. *Chest* **2004**, *125*, 1005-1011, doi:10.1378/chest.125.3.1005.
349. Radulovic, M.; Bauman, W.A.; Wecht, J.M.; LaFountaine, M.; Kahn, N.; Hobson, J.; Singh, K.; Renzi, C.; Yen, C.; Schilero, G.J. Biomarkers of inflammation in persons with chronic tetraplegia. *J Breath Res* **2015**, *9*, doi:Artn 036001

10.1088/1752-7155/9/3/036001.
350. Romero, P.V.; Rodriguez, B.; Martinez, S.; Canizares, R.; Sepulveda, D.; Manresa, F. Analysis of oxidative stress in exhaled breath condensate from patients with severe pulmonary infections. *Arch Bronconeumol* **2006**, *42*, 113-119, doi:Doi 10.1016/S1579-2129(06)60128-6.
351. Rosias, P.P.; Robroeks, C.M.; Kester, A.; den Hartog, G.J.; Wodzig, W.K.; Rilkers, G.T.; Zimmermann, L.J.; van Schayck, C.P.; Joebsis, Q.; Dompeling, E. Biomarker reproducibility in exhaled breath condensate collected with different condensers. *European Respiratory Journal* **2008**, *31*, 934-942, doi:10.1183/09031936.00073207.
352. Rosias, P.P.; Robroeks, C.M.; Niemarkt, H.J.; Kester, A.D.; Vernooy, J.H.; Suykerbuyk, J.; Teunissen, J.; Heynens, J.; Hendriks, H.J.; Jobsis, Q.; et al. Breath condenser coatings affect

measurement of biomarkers in exhaled breath condensate. *Eur Respir J* **2006**, *28*, 1036-1041, doi:10.1183/09031936.06.00110305.

353. Samitas, K.; Chorianopoulos, D.; Vittorakis, S.; Zervas, E.; Economidou, E.; Papatheodorou, G.; Loukides, S.; Gaga, M. Exhaled cysteinyl-leukotrienes and 8-isoprostane in patients with asthma and their relation to clinical severity. *Resp Med* **2009**, *103*, 750-756, doi:10.1016/j.rmed.2008.11.009.
354. Sanak, M.; Gielicz, A.; Bochenek, G.; Kaszuba, M.; Nizankowska-Mogilnicka, E.; Szczeklik, A. Targeted eicosanoid lipidomics of exhaled breath condensate provide a distinct pattern in the aspirin-intolerant asthma phenotype. *J Allergy Clin Immunol* **2011**, *127*, 1141-1147 e1142, doi:10.1016/j.jaci.2010.12.1108.
355. Shimizu, Y.; Dobashi, K.; Zhao, J.J.; Kawata, T.; Ono, A.; Yanagitani, N.; Kaira, K.; Utsugi, M.; Hisada, T.; Ishizuka, T.; et al. Proton pump inhibitor improves breath marker in moderate asthma with gastroesophageal reflux disease. *Respiration* **2007**, *74*, 558-564, doi:10.1159/000101437.
356. Sood, A.; Qualls, C.; Seagrave, J.; McDonald, J.; Shohreh, R.; Chiavaroli, A.; Schuyler, M. Effect of Allergen Inhalation on Airway Oxidant Stress, Using Exhaled Breath Condensate 8-Isoprostane, in Mild Asthma. *J Asthma* **2013**, *50*, 449-456, doi:10.3109/02770903.2013.780609.
357. Syslova, K.; Kacer, P.; Kuzma, M.; Najmanova, V.; Fenclova, Z.; Vlckova, S.; Lebedova, J.; Pelclova, D. Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases. *J Chromatogr B* **2009**, *877*, 2477-2486, doi:10.1016/j.jchromb.2009.06.008.
358. Syslova, K.; Kacer, P.; Kuzma, M.; Pankracova, A.; Fenclova, Z.; Vlckova, S.; Lebedova, J.; Pelclova, D. LC-ESI-MS/MS method for oxidative stress multimarker screening in the exhaled breath condensate of asbestosis/silicosis patients. *J Breath Res* **2010**, *4*, doi:Artn 017104

10.1088/1752-7155/4/1/017104.

359. Tanou, K.; Koutsokera, A.; Kiropoulos, T.S.; Maniati, M.; Papaioannou, A.I.; Georga, K.; Zarogiannis, S.; Gourgoulianis, K.I.; Kostikas, K. Inflammatory and oxidative stress biomarkers in allergic rhinitis: the effect of smoking. *Clinical and Experimental Allergy* **2009**, *39*, 345-353, doi:10.1111/j.1365-2222.2008.03149.x.
360. Tufvesson, E.; Bozovic, G.; Hesselstrand, R.; Bjermer, L.; Scheja, A.; Wuttge, D.M. Increased cysteinyl-leukotrienes and 8-isoprostane in exhaled breath condensate from systemic sclerosis patients. *Rheumatology* **2010**, *49*, 2322-2326, doi:10.1093/rheumatology/keq271.
361. Van Hoydonck, P.G.; Wuyts, W.A.; Vanaudenaerde, B.M.; Schouten, E.G.; Dupont, L.J.; Temme, E.H. Quantitative analysis of 8-isoprostane and hydrogen peroxide in exhaled breath condensate. *Eur Respir J* **2004**, *23*, 189-192, doi:10.1183/09031936.03.00049403.
362. Vizcaya, D.; Mirabelli, M.C.; Orriols, R.; Anto, J.M.; Barreiro, E.; Burgos, F.; Arjona, L.; Gomez, F.; Zock, J.P. Functional and biological characteristics of asthma in cleaning workers. *Resp Med* **2013**, *107*, 673-683, doi:10.1016/j.rmed.2013.01.011.
363. Wu, C.M.; Adetona, A.; Song, C.C.; Naeher, L.; Adetona, O. Measuring acute pulmonary responses to occupational wildland fire smoke exposure using exhaled breath condensate. *Arch Environ Occup Health* **2020**, *75*, 65-69, doi:10.1080/19338244.2018.1562413.
364. Zhang, J.; Zhu, T.; Kipen, H.; Wang, G.; Huang, W.; Rich, D.; Zhu, P.; Wang, Y.; Lu, S.E.; Ohman-Strickland, P.; et al. Cardiorespiratory biomarker responses in healthy young adults to drastic air quality changes surrounding the 2008 Beijing Olympics. *Res Rep Health Eff Inst* **2013**, 5-174.
365. Zhao, J.J.; Shimizu, Y.; Dobashi, K.; Kawata, T.; Ono, A.; Yanagitani, N.; Kaira, K.; Utsugi, M.; Hisada, T.; Ishizuka, T.; et al. The relationship between oxidative stress and acid stress in adult patients with mild asthma. *J Invest Allerg Clin* **2008**, *18*, 41-45.
366. Janicka, M.; Kubica, P.; Kot-Wasik, A.; Kot, J.; Namieśnik, J. Sensitive determination of isoprostanes in exhaled breath condensate samples with use of liquid chromatography-

tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **2012**, *893-894*, 144-149, doi:10.1016/j.jchromb.2012.03.005.

367. Syslová, K.; Kacer, P.; Kuzma, M.; Klusâ ková, P.; Fenclová, Z.; Lebedová, J.; Pelclova, D. Determination of 8-iso-prostaglandin F(2alpha) in exhaled breath condensate using combination of immunoseparation and LC-ESI-MS/MS. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **2008**, *867 1*, 8-14.
368. Sambiagio, N.; Sauvain, J.-J.; Berthet, A.; Auer, R.; Schoeni, A.; Hopf, N.B. Rapid Liquid Chromatography-Tandem Mass Spectrometry Analysis of Two Urinary Oxidative Stress Biomarkers: 8-oxodG and 8-isoprostane. *Antioxidants (Basel)* **2020**, *10*, 38, doi:10.3390/antiox10010038.
369. Gonzalez-Reche, L.M.; Musiol, A.K.; Müller-Lux, A.; Kraus, T.; Göen, T. Method optimization and validation for the simultaneous determination of arachidonic acid metabolites in exhaled breath condensate by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Occup Med Toxicol* **2006**, *1*, 5, doi:10.1186/1745-6673-1-5.
370. Del Rio, D.; Stewart, A.J.; Pellegrini, N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, metabolism, and cardiovascular diseases : NMCD* **2005**, *15*, 316-328, doi:10.1016/j.numecd.2005.05.003.
371. Schulte-Ladbeck, R.; Lindahl, R.; Levin, J.-O.; Karst, U. Characterization of chemical interferences in the determination of unsaturated aldehydes using aromatic hydrazine reagents and liquid chromatography. *Journal of Environmental Monitoring* **2001**, *3*, 306-310, doi:10.1039/B101354H.
372. Smith, D.F.; Kleindienst, T.E.; Hudgens, E.E. Improved high-performance liquid chromatographic method for artifact-free measurements of aldehydes in the presence of ozone using 2,4-dinitrophenylhydrazine. *Journal of Chromatography A* **1989**, *483*, 431-436, doi:https://doi.org/10.1016/S0021-9673(01)93146-2.
373. Andreoli, R.; Manini, P.; Corradi, M.; Mutti, A.; Niessen, W.M.A. Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* **2003**, *17*, 637-645, doi:10.1002/rcm.960.
374. Esterbauer, H.; Cheeseman, K.H. [42] Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. In *Methods in Enzymology*; Academic Press: 1990; Volume 186, pp. 407-421.
375. Lin, Y.-L.; Wang, P.-Y.; Hsieh, L.-L.; Ku, K.-H.; Yeh, Y.-T.; Wu, C.-H. Determination of linear aliphatic aldehydes in heavy metal containing waters by high-performance liquid chromatography using 2,4-dinitrophenylhydrazine derivatization. *J Chromatogr A* **2009**, *1216*, 6377-6381, doi:10.1016/j.chroma.2009.07.018.
376. Kartavenka, K.; Panuwet, P.; Greenwald, R.; Ehret, K.M.; D'Souza, P.E.; Barr, D.B.; Ryan, P.B. Quantification of malondialdehyde in exhaled breath condensate using pseudo two-dimensional ultra-performance liquid chromatography coupled with single quadrupole mass spectrometry. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **2019**, *1105*, 210-216, doi:10.1016/j.jchromb.2018.12.009.
377. Eurachem. Blanks in Method Validation - Supplement to Eurachem Guide The Fitness for Purpose of Analytical Methods. *First edition* **2019**.
378. Martinez-Moral, M.-P.; Kannan, K. How stable is oxidative stress level? An observational study of intra- and inter-individual variability in urinary oxidative stress biomarkers of DNA, proteins, and lipids in healthy individuals. *Environment International* **2019**, *123*, 382-389, doi:https://doi.org/10.1016/j.envint.2018.12.009.
379. Rahman, I. Reproducibility of oxidative stress biomarkers in breath condensate: are they reliable? *European Respiratory Journal* **2004**, *23*, 183, doi:10.1183/09031936.04.00131604.
380. Havet, A.; Zerimech, F.; Sanchez, M.; Siroux, V.; Le Moual, N.; Brunekreef, B.; Stempfelet, M.; Künzli, N.; Jacquemin, B.; Matran, R.; et al. Outdoor air pollution, exhaled 8-isoprostane and

current asthma in adults: the EGEA study. *European Respiratory Journal* **2018**, *51*, 1702036, doi:10.1183/13993003.02036-2017.

381. Arnett, S.D.; Osbourn, D.M.; Moore, K.D.; Vandaveer, S.S.; Lunte, C.E. Determination of 8-oxoguanine and 8-hydroxy-2'-deoxyguanosine in the rat cerebral cortex using microdialysis sampling and capillary electrophoresis with electrochemical detection. *J Chromatogr B Analyt Technol Biomed Life Sci* **2005**, *827*, 16-25, doi:10.1016/j.jchromb.2005.05.036.
382. Matsumoto, Y.; Ogawa, Y.; Yoshida, R.; Shimamori, A.; Kasai, H.; Ohta, H. The stability of the oxidative stress marker, urinary 8-hydroxy-2'- deoxyguanosine (8-OHdG), when stored at room temperature. *J Occup Health* **2008**, *50*, 366-372, doi:10.1539/joh.l7144.
383. Rosias, P.P.; Robroeks, C.M.; Niemarkt, H.J.; Kester, A.D.; Vernooy, J.H.; Suykerbuyk, J.; Teunissen, J.; Heynens, J.; Hendriks, H.J.; Jöbsis, Q.; et al. Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. *European Respiratory Journal* **2006**, *28*, 1036, doi:10.1183/09031936.06.00110305.
384. Sood, A.; Qualls, C.; Seagrave, J.; McDonald, J.; Shohreh, R.; Chiavaroli, A.; Schuyler, M. Effect of allergen inhalation on airway oxidant stress, using exhaled breath condensate 8-isoprostane, in mild asthma. *J Asthma* **2013**, *50*, 449-456, doi:10.3109/02770903.2013.780609.
385. Gonzalez-Reche, L.; Schaefer, D.; Göen, T.; Kraus, T. Validity Assessment for the Results of Three Inflammatory Markers in Exhaled Breath Condensate: A Pilot Study. **2012**, *70*.
386. Holder, C.; Adams, A.; McGahee, E.; Xia, B.; Blount, B.C.; Wang, L. High-Throughput and Sensitive Analysis of Free and Total 8-Isoprostane in Urine with Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry. *ACS Omega* **2020**, *5*, 10919-10926, doi:10.1021/acsomega.0c00661.
387. Yan, Z.; Mas, E.; Mori, T.A.; Croft, K.D.; Barden, A.E. A significant proportion of F2-isoprostanes in human urine are excreted as glucuronide conjugates. *Analytical Biochemistry* **2010**, *403*, 126-128, doi:https://doi.org/10.1016/j.ab.2010.04.016.
388. Dodig, S.; Cepelak, I. Exhaled breath condensate--from an analytical point of view. *Biochem Med (Zagreb)* **2013**, *23*, 281-295, doi:10.11613/bm.2013.034.
389. Graczyk, H.; Lewinski, N.; Zhao, J.; Sauvain, J.J.; Suarez, G.; Wild, P.; Danuser, B.; Riediker, M. Increase in oxidative stress levels following welding fume inhalation: a controlled human exposure study. *Part Fibre Toxicol* **2016**, *13*, 31, doi:10.1186/s12989-016-0143-7.
390. Loxham, M.; Nieuwenhuijsen, M.J. Health effects of particulate matter air pollution in underground railway systems – a critical review of the evidence. *Particle and Fibre Toxicology* **2019**, *16*, 12, doi:10.1186/s12989-019-0296-2.
391. Lemire, J.; Alhasawi, A.; Appanna, V.P.; Tharmalingam, S.; Appanna, V.D. Metabolic defence against oxidative stress: the road less travelled so far. *J Appl Microbiol* **2017**, *123*, 798-809, doi:10.1111/jam.13509.
392. Vinolo, M.A.; Rodrigues, H.G.; Nachbar, R.T.; Curi, R. Regulation of inflammation by short chain fatty acids. *Nutrients* **2011**, *3*, 858-876, doi:10.3390/nu3100858.
393. Bose, S.; Ramesh, V.; Locasale, J.W. Acetate Metabolism in Physiology, Cancer, and Beyond. *Trends Cell Biol* **2019**, *29*, 695-703, doi:10.1016/j.tcb.2019.05.005.
394. de Laurentiis, G.; Paris, D.; Melck, D.; Montuschi, P.; Maniscalco, M.; Bianco, A.; Sofia, M.; Motta, A. Separating smoking-related diseases using NMR-based metabolomics of exhaled breath condensate. *J Proteome Res* **2013**, *12*, 1502-1511, doi:10.1021/pr301171p.
395. Paris, D.; Palomba, L.; Mirra, V.; Borrelli, M.; Corcione, A.; Santamaria, F.; Maniscalco, M.; Motta, A. NMR Profiling of Exhaled Breath Condensate Defines Different Metabolic Phenotypes of Non-Cystic Fibrosis Bronchiectasis. *Int J Mol Sci* **2020**, *21*, 8600, doi:10.3390/ijms21228600.
396. Wang, B.; Zhang, L.; Dai, T.; Qin, Z.; Lu, H.; Zhang, L.; Zhou, F. Liquid-liquid phase separation in human health and diseases. *Signal Transduct Target Ther* **2021**, *6*, 290-290, doi:10.1038/s41392-021-00678-1.

397. Saito, M.; Hess, D.; Eglinger, J.; Fritsch, A.W.; Kreysing, M.; Weinert, B.T.; Choudhary, C.; Matthias, P. Acetylation of intrinsically disordered regions regulates phase separation. *Nature Chemical Biology* **2019**, *15*, 51-61, doi:10.1038/s41589-018-0180-7.
398. Bertini, I.; Luchinat, C.; Miniati, M.; Monti, S.; Tenori, L. Phenotyping COPD by 1H NMR metabolomics of exhaled breath condensate. *Metabolomics* **2014**, *10*, 302-311, doi:10.1007/s11306-013-0572-3.
399. Lundberg, J.O.; Weitzberg, E.; Gladwin, M.T. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nature Reviews Drug Discovery* **2008**, *7*, 156-167, doi:10.1038/nrd2466.
400. Nagababu, E.; Ramasamy, S.; Abernethy, D.R.; Rifkind, J.M. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. *J Biol Chem* **2003**, *278*, 46349-46356, doi:10.1074/jbc.M307572200.
401. Shiva, S.; Huang, Z.; Grubina, R.; Sun, J.; Ringwood, L.A.; MacArthur, P.H.; Xu, X.; Murphy, E.; Darley-Usmar, V.M.; Gladwin, M.T. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res* **2007**, *100*, 654-661, doi:10.1161/01.RES.0000260171.52224.6b.
402. Carlsson, S.; Wiklund, N.P.; Engstrand, L.; Weitzberg, E.; Lundberg, J.O. Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. *Nitric Oxide* **2001**, *5*, 580-586, doi:10.1006/niox.2001.0371.
403. Godber, B.L.; Doel, J.J.; Sapkota, G.P.; Blake, D.R.; Stevens, C.R.; Eisenthal, R.; Harrison, R. Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. *J Biol Chem* **2000**, *275*, 7757-7763, doi:10.1074/jbc.275.11.7757.
404. Ortega, R.; Bresson, C.; Darolles, C.; Gautier, C.; Roudeau, S.; Perrin, L.; Janin, M.; Floriani, M.; Aloin, V.; Carmona, A.; et al. Low-solubility particles and a Trojan-horse type mechanism of toxicity: the case of cobalt oxide on human lung cells. *Particle and Fibre Toxicology* **2014**, *11*, 14, doi:10.1186/1743-8977-11-14.
405. Gwinn, W.M.; Qu, W.; Shines, C.J.; Bousquet, R.W.; Taylor, G.J.; Waalkes, M.P.; Morgan, D.L. Macrophage solubilization and cytotoxicity of indium-containing particles in vitro. *Toxicol Sci* **2013**, *135*, 414-424, doi:10.1093/toxsci/kft154.
406. Moreno, T.; Martins, V.; Querol, X.; Jones, T.; BéruBé, K.; Minguillón, M.C.; Amato, F.; Capdevila, M.; de Miguel, E.; Centelles, S.; et al. A new look at inhalable metalliferous airborne particles on rail subway platforms. *Sci Total Environ* **2015**, *505*, 367-375, doi:10.1016/j.scitotenv.2014.10.013.
407. registry, A.f.t.s.a.d. Medical Management Guidelines for Barium (Elemental) and Selected Barium Compounds. Available online: https://wwwn.cdc.gov/TSP/MMG/MMGDetails.aspx?mmgid=321&toxid=57#:~:text=High%20level%20inhalation%20or%20oral,and%2For%20hypotension%2C%20muscle%20weakness (accessed on 23th march).
408. Il'yasova, D.; Scarbrough, P.; Spasojevic, I. Urinary biomarkers of oxidative status. *Clin Chim Acta* **2012**, *413*, 1446-1453, doi:10.1016/j.cca.2012.06.012.

ANNEXE

Literature search - Strategies

Embase.com

(('exhalation'/de OR 'breath analysis'/de OR 'smoking'/exp OR 'urine'/exp OR (Urine OR Urinary OR Urinal* OR Respiration OR EBC OR smoker* OR smoking OR ((Exhal* OR inhal* OR expir* OR inspir* OR test OR tests OR testing OR analys*) NEAR/3 (breath* OR air))):ti,ab,kw) AND ('isoprostane derivative'/exp OR '8 hydroxydeoxyguanosine'/exp OR 'malonaldehyde'/exp OR ((8-OHdg OR 8ohdg OR 8-oh-dg OR 8-ohg OR 8-OH-2dG OR 8-hydroxydeoxyguanosine OR 8-hydroxyguanine OR 8-hydroxy-g OR 8-hydroxy-dg OR 8-hydroxy-guanine OR 8-hydroxy-2*-deoxyguanosine OR F2-isoprostane* OR Isoprostane* OR Dinoprost* OR 15-f2t-isop* OR 8-epi-pgf2-alpha OR 8-epi-pgf2alpha OR 8-epi-prostaglandin*-f2alpha OR 8-epiprostaglandin*-f2alpha OR 8-epiPGF* OR 8-f2t-isoprostane* OR 8-iso-pgf2alpha OR 8-isoprostaglandin*-f2alpha OR 8-isoprostane* OR Isoprostaglandin*-f2alpha-type-iii OR prostaglandin-15-f OR Malondialdehyde OR Mda OR Tbars OR malonaldehyde):ab,ti,kw OR (('hydrogen peroxide'/exp OR (H2O2 OR (Hydrogen NEXT/1 (peroxide OR Hydroperoxide)) OR Oxydol OR Perhydrol OR Superoxol OR Hydroperoxyde):ab,ti,kw) AND ('biological marker'/exp OR 'oxidative stress'/exp OR 'reactive oxygen metabolite'/exp OR (biomarker* OR oxidant-stress OR oxidative-stress OR ROS OR ("reactive oxygen" NEXT/1 (species OR metabolite*))):ab,ti,kw))))) NOT (('juvenile'/exp) NOT ('adult'/exp OR 'worker'/exp OR 'named groups by occupation'/exp)) NOT ([animals]/lim NOT [humans]/lim)

7826	of references found	30-Apr-19
Comments :	- Filter adults : NOT (('juvenile'/exp) NOT ('adult'/exp OR 'worker'/exp OR 'named groups by occupation'/exp)) - Filter humans : NOT ([animals]/lim NOT [humans]/lim) - Without PubMed 4'280	

PubMed

("Smoking/urine"[Mesh] OR "Urine"[Mesh] OR "Respiration"[Mesh] OR "Breath Tests"[Mesh] OR Urine*[tiab] OR Urinary[tiab] OR Urinal*[tiab] OR Respiration[tiab] OR EBC[tiab] OR ((Exhal*[tiab] OR inhal*[tiab] OR expir*[tiab] OR inspire*[tiab] OR test[tiab] OR tests[tiab] OR

testing[tiab] OR analy*[tiab]) AND (breath*[tiab] OR air[tiab]))) AND ("F2-Isoprostanes"[Mesh] OR "Malondialdehyde"[Mesh] OR "8-oxo-7-hydrodeoxyguanosine"[Supplementary Concept] OR "8-epi-prostaglandin F2alpha"[Supplementary Concept] OR 8-OHdg[tw] OR 8ohdg[tw] OR 8-oh-dg[tw] OR 8-ohg[tw] OR 8-OH-2dG[tw] OR 8-hydroxydeoxyguanosine[tw] OR 8-hydroxyguanine[tw] OR 8-hydroxy-g[tw] OR 8-hydroxy-dg[tw] OR 8-hydroxy-guanine[tw] OR 8-hydroxy-2-deoxyguanosine[tw] OR F2-isoprostane*[tw] OR Isoprostane*[tw] OR Dinoprost*[tw] OR 15-f2t-isop*[tw] OR 8-epi-pgf2-alpha[tw] OR 8-epi-pgf2alpha[tw] OR 8-epi-prostaglandin*-f2alpha[tw] OR 8-epiPGF*[tw] OR 8-epiprostaglandin*-f2alpha[tw] OR 8-f2t-isoprostane*[tw] OR 8-iso-pgf2alpha[tw] OR 8-isoprostaglandin*-f2alpha[tw] OR 8-isoprostane*[tw] OR Isoprostaglandin*-f2alpha-type-iii[tw] OR prostaglandin-15-f[tw] OR 15-F2t-isoprostane[tw] OR Malondialdehyde[tw] OR Mda[tw] OR Tbars[tw] OR malonaldehyde[tw] OR (("Hydrogen Peroxide"[Mesh] OR H2O2[tw] OR (Hydrogen AND (peroxide[tw] OR Hydroperoxide[tw])) OR Oxydol[tw] OR Perhydrol[tw] OR Superoxol[tw] OR Hydroperoxyde[tw]) AND ("Biomarkers"[Mesh] OR "Oxidative Stress"[Mesh] OR "Reactive Oxygen Species"[Mesh] OR biomarker*[tiab] OR oxidative-stress[tiab] OR oxidant-stress[tiab] OR ROS[tiab] OR ("Reactive Oxygen"[tiab] AND (Species[tiab] OR metabolite[tiab])))))) NOT (("Child"[Mesh] OR "Infant"[Mesh] OR "Adolescent"[MeSH]) NOT "adult"[MeSH]) NOT (animals[mh] NOT humans[mh])

5837	**of references found**	**30-Apr-19**
Comments :	- Filter Adult : NOT (("Child"[Mesh] OR "Infant"[Mesh] OR "Adolescent"[MeSH]) NOT "adult"[MeSH]) - Filter Humans : NOT (animals[mh] NOT humans[mh])	

Central - Cochrane Library Wiley

((Urine OR Urinary OR Urinal* OR Respiration OR EBC OR smoker* OR smoking OR ((Exhal* OR inhal* OR expir* OR inspir* OR test OR tests OR testing OR analys*) NEAR/3 (breath* OR air))):ti,ab,kw) AND (("8-OHdg" OR "8ohdg" OR "8-oh-dg" OR "8-ohg" OR "8-OH-2dG" OR "8-hydroxydeoxyguanosine" OR "8-hydroxyguanine" OR "8-hydroxy-g" OR "8-hydroxy-dg" OR "8-hydroxy-guanine" OR "8-hydroxy-2-deoxyguanosine" OR "F2-isoprostane*" OR Isoprostane* OR Dinoprost* OR "15-f2t-isop*" OR "8-epi-pgf2-alpha" OR "8-epi-pgf*" OR "8-epi-prostaglandin*-f2alpha" OR "8-epiprostaglandin*-f2alpha" OR "8-epiPGF*" OR "8-f2t-isoprostane*" OR "8-iso-pgf2alpha" OR "8-isoprostaglandin*-f2alpha" OR "8-isoprostane*" OR

"Isoprostaglandin-f2alpha-type-iii" OR "prostaglandin-15-f" OR Malondialdehyde OR Mda OR Tbars OR malonaldehyde OR ((H2O2 OR (Hydrogen NEAR/1 (peroxide OR Hydroperoxide)) OR Oxydol OR Perhydrol OR Superoxol OR Hydroperoxyde) AND (biomarker* OR oxidative-stress OR oxidant-stress OR ROS OR ("reactive oxygen" NEAR/1 (species OR metabolite*))))):ab,ti,kw)

1446	**of references found**	**30-Apr-19**
Comments :		

Web of Science – Core collection*

TS=(("Urine" OR "Urinary" OR Urinal* OR "Respiration" OR "EBC" OR ((Exhal* OR inhal* OR expir* OR inspir* OR "test" OR "tests" OR "testing" OR analys*) NEAR/3 (breath* OR "air"))) AND ("8-OHdg" OR "8ohdg" OR "8-oh-dg" OR "8-ohg" OR "8-OH-2dG" OR "8-hydroxydeoxyguanosine" OR "8-hydroxyguanine" OR "8-hydroxy-g" OR "8-hydroxy-dg" OR "8-hydroxy-guanine" OR "8-hydroxy-2*-deoxyguanosine" OR "F2-isoprostane*" OR Isoprostane* OR Dinoprost* OR "15-f2t-isop*" OR "8-epi-pgf2-alpha" OR "8-epi-pgf*" OR "8-epi-prostaglandin*-f2alpha" OR "8-epiprostaglandin*-f2alpha" OR "8-epiPGF*" OR "8-f2t-isoprostane*" OR "8-iso-pgf2alpha" OR "8-isoprostaglandin*-f2alpha" OR "8-isoprostane*" OR "Isoprostaglandin-f2alpha-type-iii" OR "prostaglandin-15-f" OR "Malondialdehyde" OR "Mda" OR "Tbars" OR "malonaldehyde" OR (("H2O2" OR ("Hydrogen" NEAR/1 ("peroxide" OR "Hydroperoxide")) OR "Oxydol" OR "Perhydrol" OR "Superoxol" OR "Hydroperoxyde") AND (biomarker* OR oxidative-stress OR oxidant-stress OR ROS OR ("reactive oxygen" NEAR/1 ("species" OR "metabolite"))))) AND (human* OR adult* OR worker* OR "individual" OR "individuals" OR patient* OR "person" OR "persons" OR employee* OR "woman" OR "women" OR "man" OR "men" OR "student" OR "students"))

4330	**of references found**	**30-Apr-19**
Comments :	- Filter Humans: - (human* OR adult* OR worker* OR "individual" OR "individuals" OR patient* OR "person" OR "persons" OR employee* OR "woman" OR "women" OR "man" OR "men" OR "student" OR "students") - Non-smoking or smoker	

* The Core Collection includes the following databases: Science Citation Index Expanded (1900-present), Social Sciences Citation Index (1900-present), Arts & Humanities Citation Index (1975-present), Conference Proceedings Citation Index- Science (1990-present), Conference Proceedings Citation Index- Social Science & Humanities (1990-present), Book Citation Index– Science (2005-present), Book Citation Index– Social Sciences & Humanities (2005-present), Emerging Sources Citation Index (2015-present), Current Chemical Reactions (1985-present), (Includes Institut National de la Propriete Industrielle structure data back to 1840), Index Chemicus (1993-present)

Results

The order of import in Endnote follows the order below:

Databases and web portals	**Date**	**Number of references**	
		Found	After deduplication**
PubMed	**30.04.19**	**5837**	**5834**
Embase.com	**30.04.19**	**7826**	**3934**
Web of Science – Core collection	**30.04.19**	**4330**	**1565**
Central – Cochrane Library Wiley	**30.04.19**	**1446**	**534**
Total		**19420**	**11867**

**Method of Bramer