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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

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Highlights

- A systematic review and meta-analysis were conducted to determine the reference range of 8-OHdG in the EBC
- The null hypothesis that the 8-OHdG levels in EBC are consistent across the different control groups was strongly rejected
- The between-study variability was very high; it was mainly confounded by the analytical method and completely dominated the within-studies variability
- Smoking was evidenced as a potential determinant of 8-OHdG inter-individual variability but only when immunochemical analysis was used.
- The estimated reference values should be considered preliminary, as they are based on a limited number of studies, mostly of moderate to low quality of evidence.

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- Further research is necessary to standardize EBC sampling, storage and analytical methods.

Abstract

In the field of engineered nanomaterials (ENMs) and other airborne particulate exposure biomonitoring, circulating oxidative stress biomarkers appear promising. These biomarkers could be monitored in different biological matrices. Exhaled breath condensate (EBC) enables their measurements in the respiratory tract, without affecting airway function or creating inflammation. The 8-hydroxy-2-deoxyguanosine (8-OHdG) was found increased in the EBC of ENM-exposed workers. Our objectives were to assess the reference range of 8-OHdG in the EBC and to identify determinants of its inter- and intra-individual variability. The meta-analysis was stratified by analytical method (chemical versus immunochemical analysis) and resulted in a between-study variability over 99% of the total variability. The between-study variability completely dominated the within-studies variability. By using a mixed model with study ID as a random effect rather than a meta-regression, only smoking was evidenced as a potential determinant of 8-OHdG inter-individual variability, and only when immunochemical analysis was used. To our knowledge, this is the first meta-analysis aimed at estimating reference values for 8-OHdG in the EBC. The estimated values should be considered preliminary, as they are based on a limited number of studies, mostly of moderate to low quality of evidence. Further research is necessary to standardize EBC sampling, storage and analytical methods. Such a standardization would enable a more accurate estimation of the reference ranges of the 8-OHdG and potentially other biomarkers measurable in the EBC, which are essential for a meaningful interpretation of the biomonitoring results.

Keywords: lung inflammation; 8-hydroxy-2'-deoxyguanosine; occupational exposure; inhalation; biomonitoring

1. Introduction

Despite a paucity of epidemiological data regarding the health effects of engineered nanomaterials (ENMs) (Guseva Canu et al. 2018), both human and animal experiments reported that ENMs elicited activation of inflammatory and oxidative stress responses that could be monitored in different biological matrices as preclinical alterations (Bencsik et al. 2018; Schulte et al. 2019). Such preclinical alterations may function both as indicators of early effects before

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clinical manifestations may occur and as indirect markers of exposure, although with a low specificity for ENMs (Schulte et al. 2019). Several reports suggested the usefulness of biological monitoring to survey populations exposed to chemical hazards (Boogaard et al. 2011; Louro et al. 2019), in particular to emerging hazards with unknown toxicity, such as ENMs (Bergamaschi et al. 2015; Schulte et al. 2018; Schulte et al. 2016). Biomarkers of early adverse effects appears relevant to anticipate and indicate some negative health effects consequences that might accompany increasing production and use of ENMs in unprotected areas. They reflect early modifications preceding progressive structural or functional damage, but can be completely reversible upon the removal from the exposure of concern.

Oxidative stress (OS) is considered an important component in the toxicological pathways, leading to the expression of virtually all diseases (Frijhoff et al. 2015). OS results from an imbalance between the production of reactive oxygen species (ROS, which are natural by-products of oxygen metabolism) and the antioxidant defensive capacity of an organism (Sies 2015). Excessive production of ROS suppresses antioxidant capacity in vivo, damaging DNA, lipids, proteins, and free amino acids (Frijhoff et al. 2015). Because of the highly reactive nature of ROS and, in turn, their short half-life, measures of OS usually rely on the assessment of these products of oxidizing reactions (Chamitava et al. 2018). Despite a vast number of analytical methods developed to measure the extent and to understand the nature of oxidative stress biomarkers, their measurements in routine remains difficult. Moreover, predictive value of circulating OS biomarkers remains poorly understood and positive associations between health effects and some OS biomarkers still need to be validated (Frijhoff et al. 2015; Kander et al. 2017; Shih et al. 2019). Analytical validation is a prerequisite in biomarker development and research (WHO 2001). Furthermore, the assessments of the inter- and intra-individual biomarker variability, the kinetics of biomarker excretion, and the background biomarker values in the general population is required. The latter corresponds to the 95th percentile value of a given biomarkers in the general healthy population, while the reference range corresponds to the prediction interval between which 95% of biomarker values measured in the reference population fall into. As most biomarkers have a skewed instead of normal distribution, the reference ranges are presented as geometric mean and associated geometric standard deviation (GSD). Today, none of OS biomarkers has been validated in a large prospective cohort and very few have established reference ranges (Koutsokera et al. 2008). The absence of reference values in biological matrices other than blood is particularly salient. Nevertheless, the choice of appropriate biological matrix for a biomarker validation for non-invasive biological

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monitoring in occupational setting has a paramount importance. For ENMs, the respiratory tract is the primary route of exposure in occupational settings, thus the respiratory system plays a central role in the toxicokinetic and toxicodynamic profile of ENMs (Bencsik et al. 2018; Schulte et al. 2019). ENMs may enter other human organs and generate toxicity after they are absorbed into the bloodstream through the respiratory systems (Mu et al. 2014). As the persistence of inflammation and oxidative stress in the lungs may have a "systemic" impact on the body, other matrices could be relevant for assessing systemic inflammatory and oxidative stress response (Beard et al. 2018; Kuijpers et al. 2018; Schulte et al. 2019). 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. Instead, 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 (Andreoli et al. 2011). Therefore, the exhaled breath condensate (EBC) has been recently proposed as a non-invasively collected biological matrix to detect biomarkers of oxidative stress reflecting inflammation in the cell (Lee et al. 2015; Pelclova et al. 2016a; Pelclova et al. 2017; Vlaanderen et al. 2017; Wu et al. 2014). Consequently, EBC is the preferential matrix for establishing the reference ranges for the most promising OS biomarkers for non-invasive biological surveillance of ENM-exposed workers.

One of the most interesting oxidative stress biomarker concerns DNA damages. During the enzymatic DNA repairing process, some oxidized products are excreted from the cell without any further metabolism. One of them is 8-hydroxy-2-deoxyguanosine (8-OHdG), generated following the repair of the guanosine, the most oxidized nucleobase in ROS-mediated DNA damages guanosine (Kasai 1997). Increase in 8-OHdG level measured in the EBC was frequently reported among ENM-handling workers (Schulte et al 2019), but the absence of the reference range for this biomarkers precludes any meaningful interpretation of this finding.

The objectives of this study were to estimate the reference range of 8-OHdG in the EBC of the healthy non-smoking adults and to identify the determinants of its inter- and intra-individual variability.

2. Methods

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To achieve our objectives in a cost-effective way, we designed and conducted a systematic review and meta-analysis for several biomarkers in two matrices: urine and EBC. The protocol has been developed according to recommendations from Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher et al. 2009; Moher et al. 2015) and registered with the International Prospective Register of Systematic reviews (registration number CRD 42019124621). This study is focused on 8-OHdG measured in the EBC.

2.1. 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 (Horvath et al. 2005).

Two reviewers (MH and YS) independently performed a first screening of titles and abstracts retrieved during the searches, using Rayyan software (Ouzzani et al. 2016). 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.

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2.2. 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.

2.3. 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 (Supplementary material Table S1), 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 (Guyatt et al. 2008). 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).

2.4. Statistical analysis

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- 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 (Graille et al. (submitted)), 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 $sdL = \sqrt{\ln(1 + \frac{SD^2}{AM^2})}$

- 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

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

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

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

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:

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

with $\text{muL} = \ln(median)$, 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

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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 (Deeks et al. 2017), 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.

3. Results

3.1. Study selection

The application of the literature research string in four databases resulted in 19421 records (Figure 1). After removal of duplicates and selection of the studies where EBC was collected for analyses, 893 records remained. From these records, 590 were excluded based on the title and abstract reading. Among the remaining 303 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 (Fireman Klein et al. 2019) was excluded because only the arithmetic mean was reported - no variability (SD GSD, IC IQR) indicated. The control group in (Pelclova et al. 2016b) and in (Pelclova et al. 2016c) 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.

3.2. Descriptive results

Among the eight studies included into the systematic review, five (62.5%) were of moderate quality andthree of low quality of evidence (Supplementary material, Table S2). Among the six studies included in the meta-analyses five were analyzed chemically and one by immunochemical methods. Table S2 summarizes all the included studies and provides detailed information on their respective quality, including sub-score values for each of four domains considered.

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3.3. 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 2 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 3a). 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 3b).

4. Discussion

To our knowledge, this study is the first focusing on the 8OHdG levels measured in the EBC to estimate the reference values. This study was limited by a low number of original research studies and by the relatively poor quality of evidence gathered from these studies. A third of the studies provided low evidence, due to numerous methodological, analytical and data reporting drawbacks. We found that between-study differences were mainly confounded by the analytical method. Noteworthy, all studies originated from only two laboratories (the lab of Doruk et al. using ELISA and reporting much higher 8-OHdG levels) and the Czech laboratory using LC-ESI-MS/MS. Since these two analytical methods have different sensitivity and specificity (Carraro et al. 2010), the interpretation of these results is challenging. The LC-ESI-MS/MS has a high performance in terms of sensitivity and specificity and provides reproducible results. However, it requires expensive instruments. The ELISA depends on the commercial kits used and is less reproducible than the chemical analytical method. Therefore the analysis

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should be done in triplicate (Falk et al. 2000). Furthermore, the kit used by Doruk only covers the range 0.125-10 ng/mL, which is two orders of magnitude less sensitive than the LC-MS/MS, covering pg/ml ranges.

Despite a limited number of strata in this meta-analysis, our null hypothesis that the 8-OHdG levels in EBC are consistent across the different control groups was strongly rejected. As no details were given as to how the controls were selected, one can only speculate as to why this is so. The standard deviation (expressed in percent of the mean: the coefficient of variation) varied between 20% and 40% of the mean which seems correct and in line with within-study GSDs varying between 1.24 and 1.48. Most of the between-subgroup variability was due to the difference between the two laboratories. However even within the laboratories the between study variability exceeded by far this within-study variability leading to I-square values over 95%. Thus, the presented graphs give indications of the between-study variability within analytical method, whose origin is mainly unknown (e.g. population selection, day-to-day lab variability), but cannot claim to yield reference values for individual subjects. As gender was completely confounded with this difference, no inference can be done on its effect. With respect to smoking, non-smoking subgroups showed significantly lower level of 8-OHdG in the single study using ELISA. No difference according to smoking could be shown in the different studies of the Czech groups.

EBC could represent an ideal biological matrix for monitoring and screening of healthy individuals for possible early respiratory diseases because it enables noninvasive assessment of biochemical and inflammatory parameters in the airways. However, it still has some limitations that may explain the weakness of the database in our meta-analysis. The EBC use for analytical purposes is relatively recent and currently available analytical techniques are limited in sensitivity and specificity. Therefore, analysis of low concentration of OS biomarkers is a hurdle which partially explains the variability in reported values. Likewise, no fully validated method for calculating dilution of respiratory droplets is available (Dodig and Cepelak 2013). An ideal dilution factor would have a known and stable plasma concentration and high diffusing capacity through the cellular membrane and would not be a product of the respiratory tract (Effros et al. 2003; Konstantinidi et al. 2015). In addition, the levels of oxidative biomarkers such as the 8-OHdG have shown circadian variations (Kanabrocki et al. 2002) and can be affected by certain food, drinks or medications (Fan et al. 2000; Halliwell 2002; Kasai et al. 2001) and they are not taken into account and descripted in the studies. These still unresolved

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questions, for which we had no quantitative data, could constitute confounding factors and limit our analysis of the inter- and intra-individual variability in the 8-OHdG levels.

Standardization of EBC analysis still requires international collaborative effort. In 2005, the American Thoracic Society (ATS)/ European Respiratory Society Task Force based on the consensus of the expert panel published some recommendations for analysis of the EBC (Horvath et al. 2005) . They provide guidelines on areas of uncertainties in the expectation of optimizing future researches in their methodologies, of implementing consistent protocols and standards across research laboratories and encouraged research in this new field (Wallace and Pleil 2018). Once the EBC sampling and storage methods are standardized, along with the analytical methods, their implementation in a large representative sample of general population would enable a more accurate estimation of the reference ranges of the 8-OHdG and potentially other biomarkers measurable in the EBC.

5. Conclusion

This first attempt to determine the reference values for the 8-OHdG in the EBC using meta-analysis faced with some complex challenges. The lack of standardization of the methods currently used and the diversity of sample collection devices/ techniques impacted considerably our meta-analysis. As result, the between study variability was over 99% of the total variability and completely dominated the within-studies variability. The small number of studies precluded a comprehensive assessment of determinants of the inter- and intra-individual variability in the 8-OHdG level. Only smoking was evidenced as a potential determinant of the 8-OHdG inter-individual variability, and only when immunochemical analysis were used. To our knowledge, this is the first meta-analysis aimed at determining reference values for 8-OHdG in EBC. Values determined in this study should be considered preliminary, as they are based on a limited number of studies, mostly of moderate to low quality of evidence. Further research effort is necessary to standardize EBC sampling, storage and analytical methods. Such a standardization would enable an accurate estimation of the reference ranges of the 8-OHdG and potentially other biomarkers measurable in the EBC, which are essential for a meaningful interpretation of the biomonitoring results.

Declaration of interests

We declare that there are no known conflicts of interest associated with this manuscript and there has been no financial support for this work that could have influenced its outcome.

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Figure 1. Flow-shart of study selection

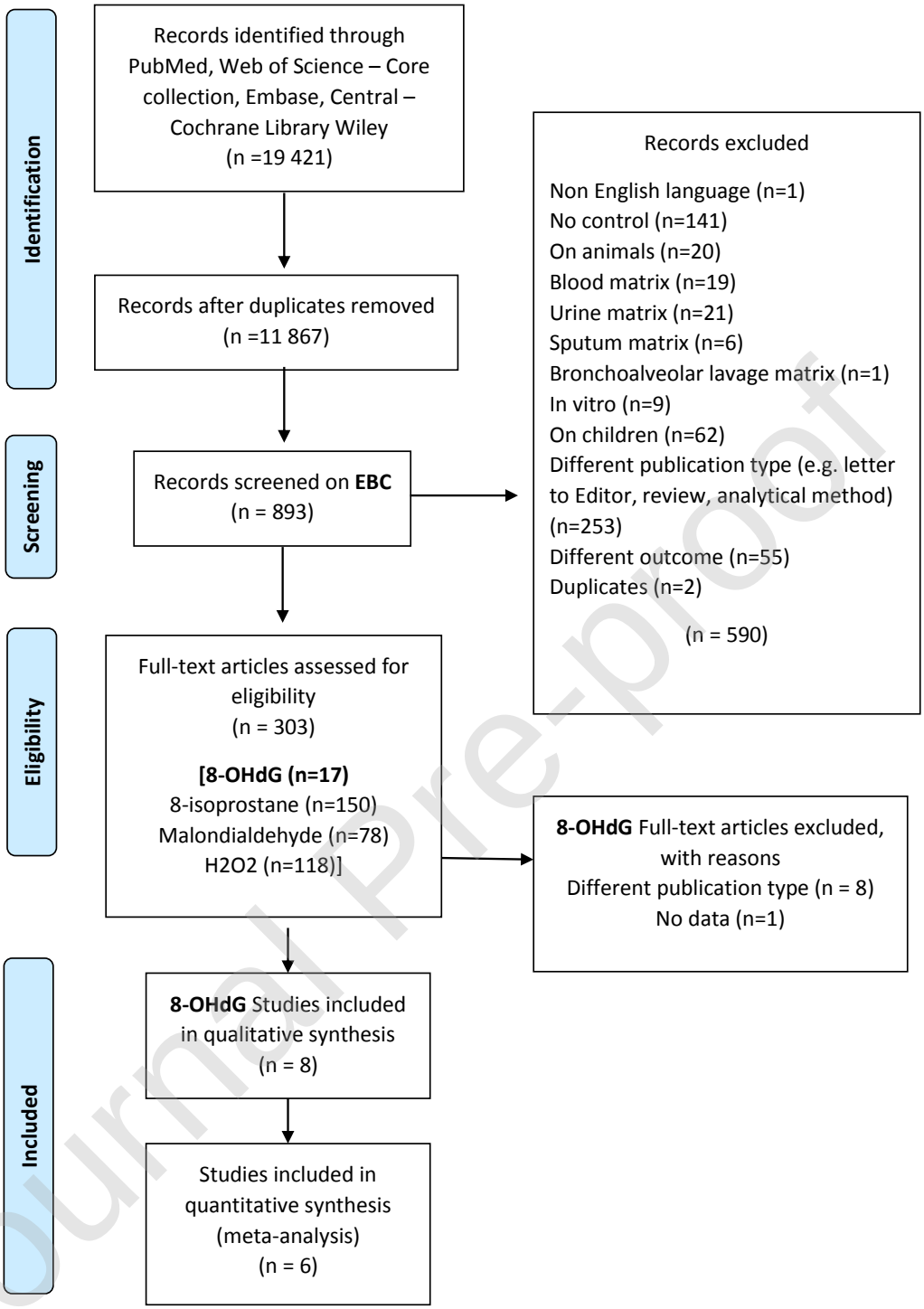

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Figure 2. Forest-plot of the 8-OHdG levels [pg/mL] measured in the exhaled breath condensate according to the analytical method

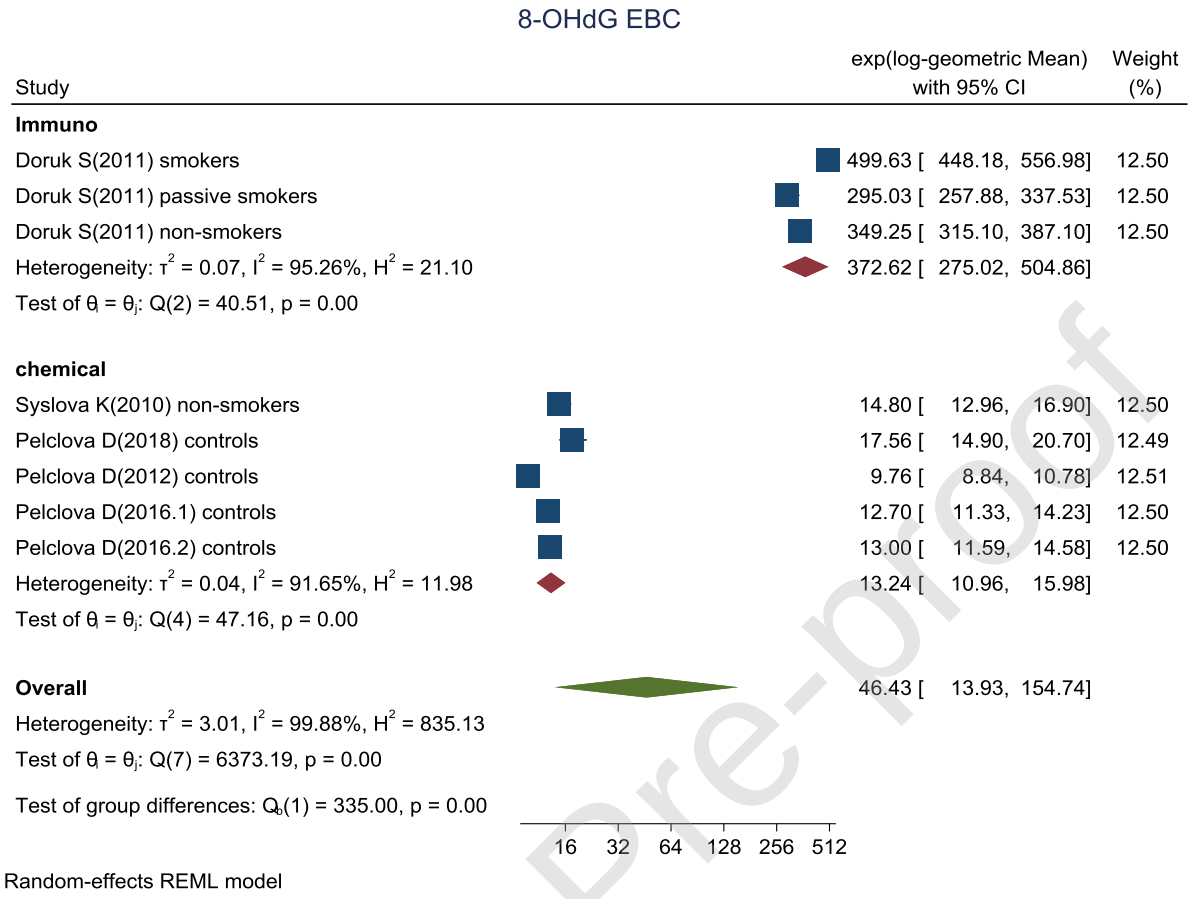

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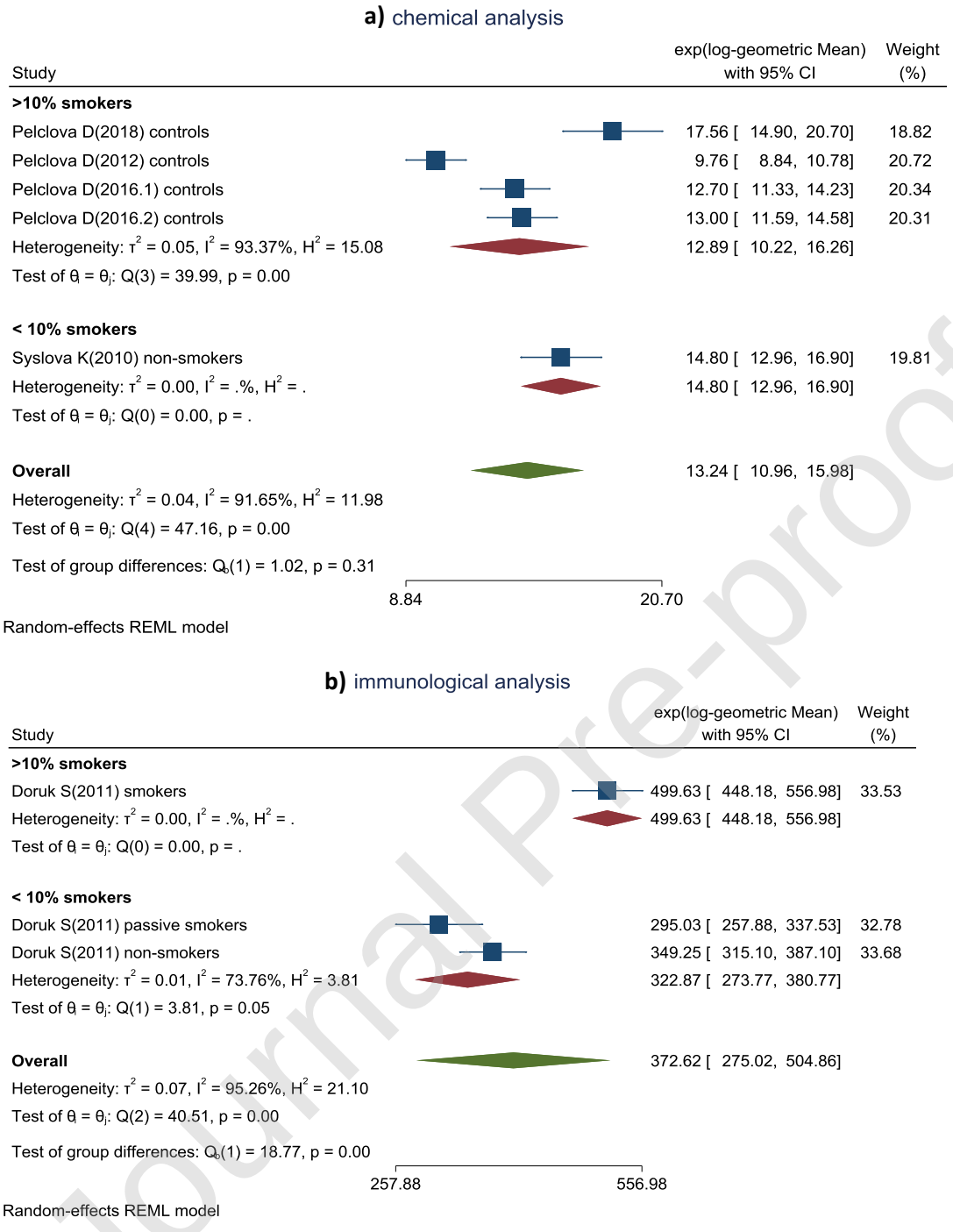
Figure 3. Forest-plot of the 8-OHdG levels measured in the exhaled breath condensate according to the smoking status and analytical method



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