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The comet assay as a tool in human biomonitoring studies: Effects of confounding factors

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ABSTRACT

The comet assay is widely used in human biomonitoring studies of environmental and occupational exposures. However, it is clear from multiple studies that various types of confounding factors might affect the direct relationship between exposure and DNA damage in the comet assay. In addition to common confounders such as age, sex, and smoking, other factors considered to be important determinants for background levels of DNA damage in the comet assay include exhaustive physical exercise, chronic diseases, medical treatment, and diet. These are typically controlled in biomonitoring studies by restriction or matching of subjects. Period effects (or seasonal variation) have been observed in a relatively large number of studies. There are various putative factors, which may cause period effects, including temporal variation in solar radiation, temperature, and air pollution. The present review describes the effects of these confounding factors in measurements of DNA strand breaks by the comet assay. In general, the literature does not indicate that any confounding factor is consistently associated with an increased level of DNA damage, measured by the comet assay. In this respect, it is important to balance the need to control for confounding with the risk of introducing in the statistical analysis a variable, which is influenced by exposure and outcome (i.e. collider bias). In addition, it is important that investigators describe procedures for controlling the effect of confounding factors in the selection of subjects and statistical analysis. Care should be taken in study design and statistical analysis to avoid unwanted effects of confounding factors.

1. Introduction

The comet assay is widely used in studies on humans to assess the effects of exposures on DNA damage and repair, measured in leukocytes and other cells [1,2]. The standard alkaline comet assay detects DNA strand breaks, alkali-labile sites and transient DNA breaks (typically shortened to DNA strand breaks). In recent years, there has been focus on technical aspects related to comet assay procedures that affect repeatability and reproducibility of results [3,4] and inter-laboratory variation in levels of DNA damage [5–9], and that have led to the development of standard procedures [10,11], and recommendations to ensure that sufficient and essential information is reported in published papers [12].

Very often, investigators highlight the ability to detect low levels of

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DNA damage as an advantage of the comet assay. The sensitivity depends on the assay protocol, but usually the comet assay can detect a difference between specimens when they differ by more than a couple of hundred lesions per human diploid cell [13]. However, what is often left out of this appraisal of the comet assay's sensitivity is that confounding factors also affect the background level of DNA damage. Indeed, assessment of exposure-outcome associations in biomonitoring studies with comet assay results entails careful control of effects of confounding factors.

The purpose of the present review is to describe the effects of confounding factors on measurements of exposure-outcome associations in studies using the comet assay. Here, a confounding factor simply refers to a host factor (e.g. age and sex) or external exposure, which can affect the background level of DNA damage. However, there are important considerations about factors that should be controlled and the best way to control these factors, as well as concerns about over-adjustment in statistical analyses. In addition, it may not be entirely clear when associations should be reported as adjusted rather than unadjusted (crude) effect sizes.

In the review, we first provide a general introduction to various categories of confounding factors and their effect on the association between exposure and DNA strand breaks. Subsequently, we describe findings on confounding factors from systematic reviews, meta-analyses on specific confounding factors, original studies of confounding factors in non-selected healthy subjects, and period (seasonal) variation in levels of DNA strand breaks. Lastly, we provide recommendations on how to report observed associations between exposure and comet assay results.

2. Methods

The present paper is part of a series of papers that assess the comet assay as a tool in human biomonitoring studies [14–20]. The papers extend the results from a scoping review of human biomonitoring studies that primarily collected and summarised results on DNA strand breaks induced by six different types of exposure [21]. Overall, the present paper is a narrative review. The discussion of studies on period effects and summary of previous reviews resemble systematic and umbrella reviews, respectively. The review encompasses results on DNA strand breaks, although we think that the effects of confounding factors can be generalised to other versions of the comet assay, such as measurements of oxidatively damaged DNA and DNA repair activity.

It is not possible to set up a specific search for studies on the effect of confounding factors in different literature databases. Therefore, we have collected information on confounding factors in previous systematic reviews on the comet assay [2,22], combined with searches in PubMed, EMBASE and Google. We have used PubMed and EMBASE because they have different indexing of keywords. Google was used for unstructured/exploratory searches. We have not performed systematic searches with specific search string of terms. For the same reason, there were not specific inclusion or exclusion criteria. We have used information from previous reviews on confounding factors (a source of general information on confounding factors for measurements of DNA damage by the comet assay) (Section 4), systematic reviews on specific confounding factors (giving effect sizes of confounding factors) (Section 5), and studies on confounding factors (including period effects) (Sections 6 and 7) in non-selected healthy humans. 'Non-selected healthy humans' refers to study populations that have not been selected as the reference group in a study on environmental or occupational exposure, lifestyle or dietary factors. The main point is that a control group in a biomonitoring study is a selected population, which may not be a representative subgroup of the population. We have included only studies on DNA strand breaks in whole blood (leukocytes), granulocytes and peripheral blood mononuclear cells (sometimes also called lymphocytes). These are typically considered to be surrogate cells, which are relatively easy to sample, and can be stored either in a fridge (days) or freezer (years) [23, 24]. In the paper, we use *leukocytes* as an umbrella term to refer to nucleated blood cells, which are detected in the comet assay. In this paper, tables contain precise information on the type of cells used in specific studies.

Effect sizes are reported as either standardised mean difference (SMD) or fold-difference. The SMD is used as the central tendency in meta-analysis of results from human biomonitoring studies when there is heterogeneity in the ways biomarkers are measured. The calculation of SMD requires information about the mean, standard deviation and number of subjects in each group. The SMD value describes the difference between exposure groups in terms of standard deviation units; thus 'SMD = 1' implies that the difference between exposure groups has the same value as the common standard deviation. We have used fold-difference as an alternative to SMD in datasets where too many studies have not reported standard deviations or there has been uncertainty about the reported standard deviations for some of the studies in the database. In the present paper, we also have calculated fold-difference in studies that have reported period effects on DNA strand breaks by the comet assay (performed in Stata with *aweight* command that weights observations as the mean from a sample of size *n*, where *n* is the weight variable).

In the text, *effect size* refers to the difference between groups of exposed subjects and controls. This may be obtained as a delta value (i.e. difference in DNA damage), fold-difference or odds ratios (or relative risks). The 95 % confidence interval (95 % CI) of these effect sizes provides information about the statistical uncertainty of the association. In contrast, the P-value does not say anything about the effect size, as even small effects can be statistically significant if the variation is low and/or the number of subjects is high.

3. Types of confounding factors

Many human biomonitoring studies use inclusion criteria for the selection of subjects. In the ideal situation, there are two variables: a predictor (exposure; independent variable) and an outcome (DNA damage; dependent variable). However, real-life exposure situations entail other variables that affect the exposure-outcome association. These additional variables might be allowed for in defining the factors used for matching exposed subjects and controls, or they might be factors that are difficult to control unless by restriction in the selection of subjects. In the description of confounders, we assume that exposures and other factors are causal agents of DNA damage. This implies that the factors are sufficient, but not necessary, causes of DNA damage. Exposures can be considered causally related to DNA damage if there is a strong association (i.e. high effect size), consistency in effects between studies, a dose-response relationship, and the effect is blunted by intervention with a protective factor. *Association* only implies a covariability between the exposure and outcome, which is not necessarily generated by a specific causal factor because it might be due to confounding.

Below, we describe examples of situations where a direct relationship between exposure and outcome variables is influenced by other variables (i.e. mediator, effect modifier, confounder or collider (see Sections 3.1–3.4 for descriptions of variables)). The examples give an impression of the complexity of the situation when the effect of confounding factors is considered in biomonitoring studies. Fig. 1 depicts situations where the association between exposure and DNA damage, measured by the comet assay, are affected by other factors. In the ideal study, there is a direct relationship between the exposure and DNA damage, and there are no other factors that affect the level of DNA damage. An unblocked connection between exposure and outcome is called a *front door path*, whereas a *backdoor path* is a connection between exposure and outcome that might be blocked (adjustment for a confounder) or unblocked (erroneous adjustment for a collider).

In general, a *variable* is a term used in statistical analyses. There are dependent (or outcome) variables and independent variables. Comet

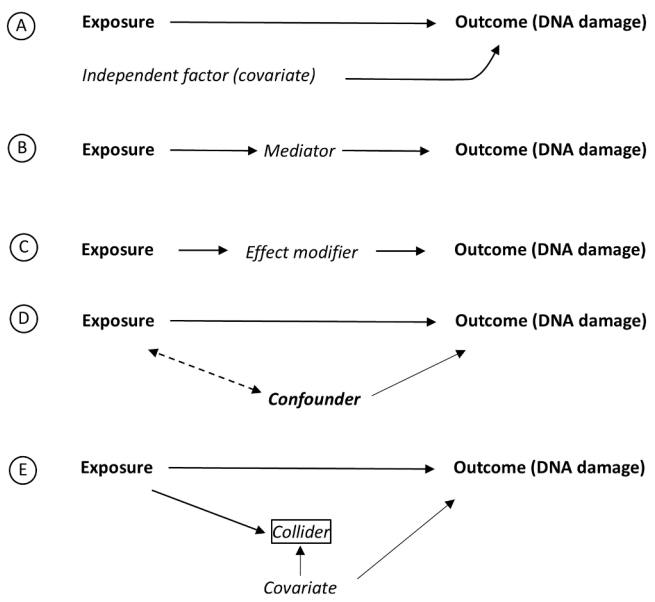

Fig. 1. Relationship between exposure and DNA damage. The association can be due to the direct effect of the predictor (A). This is indicated by an arrow between exposure and outcome (i.e. a front door path). In certain cases, covariates may lie in the front door path as mediators (B) or effect modifiers (C). Examples of covariates that are not part of the front door path are confounders (D) and colliders (E).

assay results are outcome variables in human biomonitoring studies, although they can be independent variables (i.e. predictor) in a prospective study with hard outcome (e.g. cancer). It lies in the terminology that a *factor* is an independent variable (and predictor). In the present review, we use the term *independent factor* for a predictor that is not related to the exposure, but that might affect the outcome independently of the exposure (Fig. 1**, panel A**). *Covariates* are defined as independent variables that can influence the association between the exposure and outcome in statistical tests. Therefore, covariates cover independent factors, mediators, modifiers and confounders (see below). Among independent factors (also called covariates) are unintentional/random events that might have an influence on the level of the outcome, such as unknowingly being ill at the time of blood sampling. Having data with influence from strong independent factors may affect the statistical power, and this will typically decrease the effect size of the association. Therefore, it can be relevant to include independent factors in multivariate statistical analyses as an alternative to eliminating the data as outliers.

3.1. Mediators

It is common that the exposure causes DNA damage through a mediator (Fig. 1**, panel B**). An example could be DNA damage in welders. In this case, the elevated levels of DNA damage in welders could be partly or fully mediated by chromium in welding fumes. In this case, adjusting for serum levels of chromium would decrease the effect size of the association between exposure and outcome (i.e. the front door path is blocked by adjustment for the mediator). The magnitude of attenuation reflects the relationship between direct and indirect effects of the exposure on the outcome. Although this is relevant in human biomonitoring studies, it should be emphasised that interactions between exposure and mediator, or mediator and outcome, bias the association. It is important to carefully assess any link between mediators and exposure (or outcome) to avoid statistical analyses with distorted association and flawed interpretation of the causal pathway [25].

3.2. Effect modifiers

Assessment of effect modification might be an important statistical analysis in a human biomonitoring study to understand the underlying cause(s) of variability in the outcome (Fig. 1**, panel C**). There is always a solid biological mechanism related to effect modification. Examples of effect modifiers include polymorphisms related to metabolic activation of xenobiotic compounds or differences in DNA repair capacity. Age and sex might also be considered as effect modifiers. Ideally, biomonitoring studies are designed to test the effect modification by selecting subjects according to both exposure and effect modifier (e.g. 2×2 factorial design). Statistical analysis entails an assessment of the interaction between exposure and effect modifier.

3.3. Confounders

Per the textbook definition, a classic confounder is a factor that is associated with exposure as well as being a causal factor for the outcome (Fig. 1**, panel D**). This is a backdoor path that closes by controlling for the confounder. In the context of the comet assay, a confounder is a factor that is believed to cause DNA damage. It is important to note that confounders might be controlled by the study design or statistical analysis of the results. However, statistical analysis of data in a well-designed study may be confounded. For instance, a study on occupational exposure may adequately control for age by matching exposed and unexposed workers, but the statistical analysis includes the duration of exposure as a variable in the exposed group. This will be confounded by the age of the subjects (i.e. there is an association between age and duration of exposure, and age is a predictor of DNA damage). In addition, different study designs may have different types of confounders. For instance, a cross-sectional study should control for smoking as a confounder, whereas it is not a problem in a panel study because subjects are their own control. On the other hand, panel studies are vulnerable to confounding by uncontrolled factors that vary over time (e.g. seasonal variation).

It is important to emphasise that controlling for confounding factors is a non-productive procedure because its purpose is to eliminate the effect of independent risk factors. Controlling confounders typically eliminates the possibility of comparing the effect of important independent factors. For instance, a study on air pollution exposure might be designed to control for smoking by restricting the inclusion of subjects to non-smokers. However, there is a downside to this approach because the effect of air pollution cannot be compared to the effect of smoking (i.e. a well-known genotoxic exposure), and it misses the opportunity to assess the effect modification of smoking on air pollution-generated DNA damage. It should also be noted that controlling for confounding by restriction affects the generalizability of the results.

3.4. Colliders

The influence of colliders has gained increased attention as an issue of concern in epidemiology. Typically, it has been fuelled by counter-intuitive observations as for instance the “obesity paradox” (i.e. patients with heart failure have an increased chance of survival if they are obese), which can be explained by an unfortunate combination of conditioning on an intermediate effect and unmeasured risk factors [26].

A collider is a common factor that is linked to two putatively independent predictors. The arrow heads in a causal diagram point toward the collider **(**Fig. 1**, panel E)**. Collider bias can be introduced by controlling for a variable that occurs after exposure. It may also occur in the statistical analysis if researchers have included two covariates as putative confounders, but one is actually dependent on the exposure, and the other is causing the outcome. In these cases, there will be a distorted association between exposure and outcome because the statistical analysis has linked two unrelated variables. Controlling for the putative confounder has opened a backdoor path. The collider bias phenomenon

was originally described as a way of selection bias when subjects are stratified on the collider (typically called collider stratification bias) [27]. However, it is becoming more common to distinguish between collider stratification bias (related to selection bias) and collider adjustment bias (related to statistical analysis) [28]. Inclusion of a collider into the statistical model creates a spurious association between the exposure and outcome. Thus, the consequence is a distorted association due to over-adjustment in the statistical analysis.

4. Reviews on confounding factors in the comet assay

The literature on the comet assay in human biomonitoring studies is blessed with numerous reviews. Some of these reviews have investigated the effect of exposure to specific chemicals on multiple biomarkers of DNA damage, which makes discussions about confounding factors somewhat more general (not included in the present review). A number of reviews have focused only on the comet assay as a tool in human biomonitoring studies and have therefore addressed issues that pertain specifically to the comet assay. Below is a summary of the reviews from 2000 to 2024, which provide insight into the views on the effect of confounding factors on comet assay results.

The comet assay has been used in biomonitoring studies since the early 1990's and during that decade, there was a substantial accumulation of literature on exposures associated with increased levels of DNA strand breaks. By 2000, age, sex, smoking, physical exercise, air pollution, residential radon, infection, diet (mainly antioxidants) and season (sunlight) were highlighted as factors that could affect the background levels of DNA strand breaks in human leukocytes [29]. It was noted that many of the studies tested the effect of only one factor on background levels of DNA strand breaks, but the factors were rarely statistically significant in cross-sectional studies with assessment of more than one independent factor [29].

In 2000, Albertini and co-workers published guidelines for the monitoring of genotoxic effects in humans, including techniques such as the comet assay [30]. Specifically, for the comet assay, the authors considered age, sex, smoking, recreational/medical drug use, health status and recent anaerobic exercise as important information to collect as study population characteristics. The authors highlighted the possibility of exploring effect modification in stratified statistical analyses, although they also highlighted the need to analyse associations by multivariate methods. Moreover, multiple comparisons were flagged as a concern related to false positive associations in statistical analysis [30]. Specific tests were not suggested by the authors, but Bonferroni correction and false discovery rate would be the tests of choice today to compensate for multiple testing.

In 2004, Faust and co-workers conducted a literature review on human biomonitoring studies and considered age, sex, smoking, alcohol consumption, and genetic polymorphisms (i.e. cytochrome P450 and glutathione S-transferase gene polymorphisms) as important factors to consider in human exposure studies [31]. Interestingly, the authors linked mixed results on DNA strand breaks by smoking (i.e. a well-known genotoxic agent) and mostly positive results of pesticide exposure (assumed at that time to be non-genotoxic agents) to uncontrolled confounding factors [31].

In 2009, Valverde and Rojas conducted a large review of biomonitoring studies on comet assay endpoints [32]. On the issue of confounding factors, the authors highlighted the importance of identifying differences between exposed and control groups with respect to lifestyle variables (e.g. diet, smoking habits, medical treatments, history of chronic diseases, and physical activity). However, the authors noted that negative/inconclusive results have been obtained in studies on smoking, which was speculated to be due to differences in exposure time, number of cigarettes smoked per day and the kind of tobacco smoked [32].

The ComNet project was launched as a multi-laboratory effort to investigate whether the comet assay is a reliable biomarker assay which can be used in human biomonitoring. By 2014, the project members published a status article on the comet assay in human biomonitoring that highlighted age, sex, smoking, drinking habit, exercise and drug intake as important confounding factors [22]. The ComNet project was superseded by the hCOMET COST Action and a group of authors published in 2020 a comprehensive review of the comet assay in human biomonitoring [2]. Importantly, the authors categorized confounding factors in four groups as (i) intrinsic and non-modifiable (age, sex and prescribed medication), (ii) intrinsic and modifiable (tobacco smoke, occupational exposure, obesity, over the counter drugs), (iii) extrinsic and non-modifiable (air pollution, ambient temperature, sunlight), and (iv) extrinsic and modifiable (diet, physical activity, alcohol consumption) [2]. Further to this categorisation, the authors attempted to bring a bit of terminology into the discussion by highlighting the difference between confounders, effect modifiers and mediators [2]. Within the hCOMET project, Milic and co-workers collected original data from 105 studies, which had been conducted by 44 laboratories in 26 countries ($n = 19{,}320$ results) [33]. A pooled analysis of the data indicated a positive association between age and DNA strand breaks, whereas there was no effect of sex or smoking habit [33].

In 2023, Misik and co-workers published a comprehensive review of human intervention studies on dietary factors and considered also various confounding factors [34]. The authors highlighted a number of factors that potentially have an impact on the outcome of the intervention by either causing confounding or effect modification (i.e. age, sex, smoking, alcohol consumption, body mass, lifestyle, physical activity, food supplements and diseases) [34]. In addition, seasonal differences were highlighted as a factor that might affect the reproducibility of intervention studies. Such seasonal differences were attributed to solar radiation and outdoor air pollution from traffic or wildfires [34].

Collectively, the previous reviews of biomonitoring studies have consistently highlighted age, sex, smoking, and physical exercise (typically exhaustive exercise) as confounding factors that should be controlled. Other factors are less consistently mentioned, including alcohol consumption, air pollution, diet and season (or factors that have seasonal variation, such as solar radiation and temperature). It is interesting to note that the list encompasses factors that are generally considered to be confounders (age, sex and smoking), and other somewhat unusual factors (exercise, season, and air pollution). The list of potential confounders probably reflects the background of researchers who have written the reviews.

5. Meta-analyses of confounding factors

A few systematic reviews have assessed the effect and reproducibility of specific confounding factors. Table 1 shows the overall results from meta-analyses on DNA strand breaks in leukocytes from humans. Interestingly, it can be seen that meta-analyses focused on age and sex show relatively low effect sizes [35]. Outdoor air pollution and smoking habit have somewhat higher effect sizes [36,37]. A recent systematic review of biomonitoring studies also indicates a positive association between indoor air pollution and DNA strand breaks [38]. Exhaustive physical exercise is associated with even higher effect size, although it should be emphasised that the studies are based on long-distance running or controlled laboratory experiments with subjects performing standardized exercise tests [39]. The influence of period (seasonal) effects on levels of DNA strand breaks is included in the table and further discussed below (Section 7). It appears that the period effect size (high versus low) is higher than classic confounders such as age, sex and smoking. The highest effect sizes have been obtained in studies on patients with chronic diseases [40].

Table 1
Effect of confounding factors on levels of DNA strand breaks in meta-analyses.

Confounding factor	Number of studies (subjects)	Standardised mean difference (95 % CI)	Fold difference (95 % CI)	Reference
Age	16 (2530 all age groups)	Not reported	1.01 per year (1.09, 1.11)[a]	[35]
Sex (men vs. women)	13 (1151 men/1385 women)	−0.14 (−0.41, 0.13)[b]	1.08 (1.04, 1.17)[a]	[35]
Air pollution	16 (1003 exposed/697 referents)[c]	0.56 (0.37, 0.76)	1.51 (1.28, 1.73)	[36]
Smoking	37 (803 smokers/ 959 non-smokers)	0.55 (0.16, 0.93)	Not reported	[37]
Exercise				[39]
-Overall	15 (203)	1.14 (0.67, 1.61)[d]	Not reported	
-High-intensity (>75 % $VO_{2\text{-max}}$)	5 (69)	1.57 (0.94, 2.20)[d]	Not reported	
-Long distance (≥42 km)	6 (89)	0.44 (−0.22, 1.09)[d]	Not reported	
Period (seasonal) effect	15 (2400 samples)[e]	Not reported	2.04 (1.44, 2.65)	This paper
Diseases[f]				[40]
- Coronary artery disease	13 (922 patients/817controls)	1.57 (1.06, 2.09)	2.38 (1.80, 2.95)	
- Diabetes	25 (954 patients/806 controls)	1.31 (0.61, 2.01)	2.01 (1.66, 2.55)	
- Renal disease	14 (1038 patients/895controls)	1.22 (0.98, 1.46)	2.08 (1.50, 2.67)	
- Breast cancer	15 (700 patients/727controls)	1.20 (0.68, 1.72)	3.60 (1.91, 5.29)	

[a] Based on linear regression analysis, weighted for the number of subjects in the individual studies. The original values have been converted from percent change to fold values.
[b] Forest plot of the standardised mean difference is reported in Supplementary Figure S1.
[c] Forest plot of the standardised mean difference is reported in Supplementary Figure S2.
[d] The 95 % CI has been estimated from graphs (Forest plots). Assessment of time course effect suggests the strongest effect is seen at 24 h post-exercise (SMD = 2.47, 95 % CI: 1.32, 3.61).
[e] The number corresponds to samples rather than subjects because some results originate from panel studies with repeated sampling on the same subjects. Plot of effect vs publication year is reported in Supplementary Figure S3.
[f] Alzheimer's disease, chronic obstructive pulmonary disease and lung cancer were assessed in the meta-analysis as well, but there are relatively few studies.

6. Effect of confounding factors in studies on non-selected healthy subjects

Many biomonitoring studies on environmental or occupational exposures have assessed the effects of confounding factors in post-hoc analyses, of either the control group or all exposure groups. Generalisation of effects of confounding factors from these exposure studies might be biased because it is based on data from selected groups of the population (i.e. a type of collider bias). Even subjects in the control group have been selected to match the subjects in the exposure group. Thus, subjects in the control groups are similar to the exposure groups, but they are not necessarily a subsample of the normal healthy population. Studies with subjects that are not matched controls to specific exposures are preferred for investigations of the effects of confounding factors. Such subjects are unselected in the sense that they do not need to fulfil certain criteria of confounding factors, although there might be other reasons for sampling a narrower group of subjects than the general population.

Table 2 summarises results on confounding factors for background levels of DNA strand breaks in cross-sectional and panel studies of unselected healthy subjects. In this summary, we have only included studies with at least three confounding factors, and results have been analysed by parametric statistical tests. The studies in Table 2 have been published between 1995 and 2024, but they appear to have been conducted in two different periods (9 papers in 1995–2006 and 6 papers in 2014–2024). Interestingly, only one study reported an effect of age on background levels of DNA strand breaks, and this study appears to have been designed for the purpose of assessing age specifically because the results are stratified into age groups [41]. Differences between sexes have been assessed in 12 studies, of which 9 show no differences [24,41–48]. Two studies showed that men had a higher level of DNA strand breaks than women [49,50], whereas another study showed the opposite effect [51]. The effect of smoking as a confounding factor is somewhat puzzling. It was detected as a factor with positive association with DNA strand breaks in early studies [42–44,52], but it has not been a factor with statistical significance in later studies [41,46–49,53]. There does not appear to be a consistent effect of alcohol consumption (5 studies out of 6 have shown no association between alcohol intake and DNA damage) [24,43,46,47,49,53]. However, a fairly large number of studies have detected period (or seasonal effects), and it has been consistent over the period from 1995 to 2024 [24,42,44,45,47,48]. The period (seasonal) effect is discussed in further detail below. Other factors have not been assessed consistently in the studies.

7. Sampling period (period effects)

A period effect implies that there is a systematic variation in DNA damage level over time, which affects the whole (or a large part) of the study population. It is sometimes called seasonal variation, which suggests that exposures differ during seasons, but this is somewhat misleading because period effects can occur within seasons too. Above all, period effects are true biological responses to external exposures. The most common external exposures that have been linked to period effects are solar radiation, temperature, outdoor air pollution and dietary (antioxidant) factors. Increasing background levels of DNA strand breaks from the North to the South of Europe have also been hypothesised to be related to sunlight exposure and ambient temperature (i.e. factors that depend on the latitude), whereas there was no association related to the longitude [54]. Table 3 shows a summary of studies that have assessed period (seasonal) effects in studies on healthy humans.

It is puzzling that the first reports of period effects in the comet assay are covered in a cloud of obscurity. The first publication, a cross-sectional study on healthy subjects in Pisa (Italy), merely reported "sampling month" as a significant effect in tables with statistical output [55]. However, it is not possible to ascertain whether sampling month refers to calendar month or the time after the start of sampling. A follow-up study from the same group showed the same effect, and although results were stratified by sampling month, the authors did not link it to calendar month [42]. In fact, only in the third paper from the same group was it stated that the highest level of DNA strand breaks was observed in summer months [56]. At the same time, another group in the Czechia had observed a period effect, although it was only a sampling period from October to February, and the authors considered it to be related to outdoor air pollution [57]. A study from Uppsala (Sweden) assessed DNA strand breaks over a 20-week period (February to June); the authors noted that the levels of DNA strand breaks were "fairly stable" in lymphocytes [58]. However, inspection of the reported data suggests a low level of DNA strand breaks in the first part of the study

Table 2
Effect of confounding factors on levels of DNA strand breaks in cross-sectional studies of non-selected healthy subjects.

Study design and population	Subjects (men/women)	Sample	Comet descriptor	Predictors[a]	Reference
Cross-sectional study (Italy)	200 (85/115, 10–85 years)	Lymphocytes (fresh)	Comet length	Age, sex, **smoking**, **sampling month**	[42]
Cross-sectional study (India)	62 (62/0, 23–57 years)	Lymphocytes (fresh)	TL, TI, TM	Age (equivocal), **smoking**, **diet** (vegetarian vs non-vegetarian) [statistics only one-way ANOVA and separate analysis for age]	[52]
Cross-sectional study (India)	230 (124/106, 20–30 years; university students)	Lymphocytes (fresh)	TL, TI, TM	**Sex** (men>women), smoking (including alcohol consumption), diet (vegetarian vs non-vegetarian) [statistics only include multiple *t*-tests]	[49]
Panel study of non-smokers (Denmark)	21 (7/14, 26–59 years), repeated sampling throughout 14 months	PBMC (fresh)	TM	Age, **sex** (women>men), **sunlight influx**, **hours in the sun**, smoked/fried meat, vitamin pills, fresh fruits, coffee, being ill (seven days before sampling) and exercise (three days before blood sampling)	[51]
Cross-sectional study (Austria)	80 (40/40, 21–60 years)	Whole blood (fresh)	VS (5-class)	**Age**, sex, smoking[b]	[41]
Cross-sectional (Korea)	109 (66/43, 20–28 years; university students)	Lymphocytes (fresh)	TL, TM	Sex, **smoking**, BMI (WHR), alcohol consumption, coffee, tea and exercise (hours/day)[c]	[43]
Cross-sectional study (Sweden)	99 (49/50, 19–31 years; university associated, non-smokers)	PBMC (frozen)	TL, TI, TM	Age, **Sex** (men>women), **fruit intake** (inverse in women), BMI, physical activity, number of colds in recent year, place of residence (univariate analysis, no adjustment for multiple comparisons)	[50]
Cross-sectional study (Croatia)	170 (94/76, 20–64 years)	Whole blood (fresh)	TL	Age, sex, **smoking**, **season**, **medical exposure** (especially diagnostic X-rays)	[44]
Cross-sectional (Italy)	79 (28/51, 24–80 years)	PBMC (fresh)	TI	Age, sex, **season (temperature, solar radiation)** and air pollution (ozone)	[45]
Cross-sectional (Denmark)	993 (387/605, 18–93 years; national survey volunteers)	PBMC (frozen)	VS (5-class)	Age, sex, smoking, BMI (WHR), blood pressure, cholesterol, triglycerides, HbA1c, CRP, alcohol, fish, fruits, salad, whole grains, vegetables, potatoes[d]	[46,80]
Blood donors (Poland)	276 (158/118, 18–57 years)	Whole blood (fresh)	VS (5-class)	Sex, smoking, alcohol consumption, medicines and radiological investigation within the past year, **season** (summer highest) and **pet ownership** (multivariate analysis; age not included)	[47]
Cross-sectional study (general population, Croatia)	162 (48/114, 39 ± 13 years)	Whole blood (fresh)	TL, TI, TM (either as mean or median)	Age, sex, smoking, BMI, **sampling season**, **diagnostic radiation exposure** (1 year to 1 month before sampling)	[48]
Cross-sectional study (Croatia)	60 (60/0, 18–66 years)	Whole blood (frozen)	TL, TI, TM	Age, smoking, BMI and alcohol consumption	[53]
Cross-sectional study (Brazil)	514 (492/23, 18–64 years)	Whole blood (fresh)	TL, TI	Age, **radiation exposure**, income, *Chimarao* consumption[e]	[81]
Cross-sectional study (general population, Croatia)	373 (172/201, 38 ± 9 years)	Whole blood (fresh and frozen)	TL, TI, TM (either as mean or median)	Age, sex, **smoking**, **alcohol**, **season**[f]	[24]

Abbreviations: body mass index (BMI), C-reactive protein (CRP), haemoglobin A1C (HbA1c; glycosylated haemoglobin), peripheral blood mononuclear cells (PBMC), tail intensity (TI; percent fluorescence in the comet tail), tail length (TL), tail moment (TM), visual score (VS), waist-hip ratio (WHR).

[a] Predictors in bold text have been statistically significant in the original studies.

[b] The effects of age, sex and smoking have been calculated by linear mixed model by the authors of the present review, based on raw data in the publication. Age is included as a continuous variable on four different age groups (0, 1, 2 and 3) because the results were reported in age groups rather than the age of each individual in the study.

[c] Associations are mainly reported as Pearson correlation coefficients, although the authors also refer to analyses with adjustment and "partial correlation". It is uncertain if these associations are based on two-factor or multivariate analysis. In any case, the results appear to show an effect of smoking in adjusted analyses and weaker associations by other factors are not statistically significant in adjusted analyses.

[d] The study showed effects on oxidatively damaged DNA (Fpg- and hOGG1-linked comet assay) on various parameters, including cholesterol, triglycerides, HBA1c, alcohol intake, fish and salad.

[e] Positive association with radiation exposure and tail length in multiple linear regression analysis. However, there is no association by tail intensity (regression coefficient and p-value are not reported). Information on other relevant predictors has been obtained, but they have apparently not been included in the multiple regression analysis (e.g. smoking and alcohol consumption). Descriptive data on smoking and alcohol consumption indicate very similar levels between never, ever and current users.

[f] Effect based on full dataset with fresh and frozen samples.

(tail inertia ≈ 75 in February) and high levels in the last part of the study (tail inertia ≈ 290 in June; results are estimated from graph and statistical analysis of a period effect was not done in the original publication) [58]. Unpublished results from a biomonitoring study on subjects in the US were also known by comet assay researchers in the late 1990s, which showed higher levels of DNA strand breaks in leukocytes sampled in the summer as compared to the winter (the results were shown in a talk by Tice at the Comet assay workshop in Cryodon, UK in 1997 [59] and mentioned by Møller et al., 2000 [29] and Verschaeve et al., 2007 [60] in publications on seasonal variation of DNA strand breaks in leukocytes). A fourth group of researchers also stumbled on a period effect in a study that was carried out in Copenhagen (Denmark) between March and May 1996 [61]. The authors had noticed that the weather had changed from winter (cold) to spring (warm/sunny) over the study period and therefore considered solar radiation to be a contributing factor. In fact, both the level of DNA strand breaks (comet assay) and DNA repair activity (unscheduled DNA synthesis) were positively correlated with solar radiation [61]. A follow-up study from the same group analysed blood samples over 14 months, covering two summer periods [51]. As compared to the previous study, it had a number of improvements to more firmly link the association between solar radiation and DNA strand breaks, namely, the time spent in sunlight correlated with the level of DNA strand breaks, and assay controls did not demonstrate the same type of period effect [51]. However, it does not rule out confounding by other factors that co-vary with sunlight exposure. In fact, a study from another Danish laboratory showed a period

Table 3
Relationship between period effects (variation over time) and levels of DNA strand breaks in human leukocytes.

Country	Subjects	Sampling period	Cell	Comet descriptor	Effect	Reference
Pisa (Italy)	100 (MF, NS/S)	Not reported	Lymphocytes (fresh)	Comet length	Sampling month [not further clarified]	[55]
Pisa (Italy)	200 (MF, NS/S)[a]	11 months (dates not reported)	Lymphocytes (fresh)	Comet length	Sampling month [highest in summer month][b]	[42]
Pisa (Italy)	90 (MF, former smokers)	12 months (March 1995 to March 1996)[c]	Lymphocytes (fresh)	Comet length	Sampling month (highest level in June)	[56]
Teplice (Czechia)	19 (F)	Oct 25th 1993 to Feb 1st 1994 (four sampling days)	Lymphocytes (fresh)	TI, TM	Highest 30th Nov [considered to be related to outdoor air pollution]	[57]
Uppsala (Sweden)	5 (M)	20 weeks (Feb 1995 to June 1995)	Lymphocytes (fresh)	Tail inertia	Reported as being low and fairly stable levels of DNA damage [assessment of results suggests an increase during the study period].	[58]
Copenhagen (Denmark)	62 (MF)	March to May 1996	PBMC (fresh)	TM	Highest in the second part of the study. Correlation between solar radiation and DNA damage.	[61]
Bratislava (Slovakia; panel study)	11 (M, S/NS)	October 1996 to September 1997	PBMC (fresh)	VS (5-class)	Highest during the winter (coldest; January to March) month compared to summer (warmest; July to September) month [sensitive analysis in Supplementary Table S1 suggests period effect is ambiguous].	[64]
Copenhagen (Denmark; panel study)	21 (MF, NS/S)	May 1997 to July 1998	PBMC (fresh)	TM	Highest during summer months. Correlation with solar radiation and hours spent in the sun.	[51]
Copenhagen (Denmark; panel study)	47 (MF, NS)	Nov 1999 to August 2000	PBMC (fresh)	VS (5-class)	Highest during summer (association between outdoor temperature and DNA damage).	[62]
Athens (Greece; panel study)	40 (M, NS)	March and September	PBMC (fresh)	TI	Higher level in summer (September) than winter (March).	[63]
Florence (Italy)	79 (MF, NS/S)	12 months (winter, spring, summer and autumn)	PBMC (fresh)	TI	Highest level in summer. Best correlation with outdoor temperature; less convincing correlation with solar radiation and outdoor ozone levels. Assay controls do not indicate a difference between seasons.	[45]
Zagreb (Croatia)	170 (MF, NS/S)	12 months (stratified into winter, spring, summer and autumn)	Leukocytes (fresh)	TL	Highest level in samples obtained in the winter period compared to summer [sensitive analysis in Supplementary Table S1 suggests period effect is ambiguous].	[44]
Mol (Belgium; panel study)	45 (not reported)	June, August, November, February	Leukocytes (fresh)	TI	Highest levels in June/August. Levels of DNA damage correlated with both outdoor ozone and temperature.	[60]
Zagreb (Croatia)	162	Winter (December-February), Spring (March-May), Summer (June-August) and autumn (September-November)	Whole blood (fresh)	TI, TI, TM	Highest levels in summer. Positive correlation with global sun radiation, outdoor temperature and insolation.	[48]
Warsaw (Poland)	276 (MF, NS/S)	Summer, autumn and winter (months not specified)	Whole blood (fresh)	VS (5-class)	Highest level in samples from summer, followed by autumn and winter.	[47]
Zagreb, Slavonski Brod and Vinkovci (Croatia)	373 (MF, NS/S)	Summer and winter	Whole blood (fresh and frozen)	TL, TI, TM	Highest levels in summer.	[24]

Abbreviations: F: females, M: males, NS: non-smokers, S: smokers, tail intensity (TI; percent fluorescence in the comet tail), tail length (TL), tail moment (TM), visual score (VS).
[a] Contains also the 100 subjects from Betti et al., 1994 [55].
[b] A later article from the same group of authors stated that the highest level of DNA damage was observed in "summer months".
[c] Information from materials section. Information in a figure legend states that it was March 1994 to March 1995.

effect that correlated with outdoor temperature [62].

Higher levels of DNA strand breaks in samples from the summer months have also been seen in studies from Athens (Greece) [63], Florence (Italy) [45], Mol (Belgium) [60], Zagreb (Croatia) [24,48], and Warsaw (Poland) [47]. However, it should be noted that a few studies have observed higher levels of DNA strand breaks in samples that were collected in the winter. A panel study on 11 middle-aged men (analysed once a month for 12 months) showed higher levels of DNA strand breaks in the warm months (July to September) as compared to the cold months (January to March), which was hypothesized to be related to seasonal variation in the intake of fruits and vegetables [64]. Another study from Zagreb (Croatia) showed higher levels of DNA strand breaks in samples that were collected during the winter as compared to the summer period [44]. Both of these studies used the period from January to March as cold/winter months and July to September as warm/summer months. Other researchers from the same area have used December to February and June to August as cold/winter and warm/summer months, respectively. Interestingly, if the same categorisation is used for the studies by Dusinska et al. [64] and Kopjar et al. [44], the seasonal effect is much smaller (possibly statistically non-significant) (Supplementary Table S1 shows a sensitivity analysis of the original and alternative categorisation of seasons). Taking this into consideration, there might be a less clear effect of cold/winter months in these studies.

Collectively, there is evidence to suggest that period effects are an issue in human biomonitoring studies with the comet assay. However, it is also puzzling that the observations all come from studies in Europe, with the exception of the unpublished results from the US. It is unclear if period effects are not an issue in other areas where the comet assay is often used in biomonitoring studies, or if it has just not been investigated.

Table 4
Gene-environment interaction in studies on environmental/occupational exposure to genotoxic compounds and polymorphisms in glutathione S-transferase genes.

Exposure	Number of subjects	GSTM1[a]	GSTT1[a]	GSTP1[a]	Reference
Air pollution (benzene)	135	No	No	No	[82]
Air pollution (PAH)	100	*Wildtype*	No	No	[83]
Air pollution (PAH)	65	No	No	No	[84]
Diesel engine exhaust	123	No	*Null*	NR	[85]
Coal tar (PAH)	115	No	No	NR	[86]
Coke oven (PAH)	240	No	No	NR	[87]
Glass fibres	116	No	No	No	[88]
Fibres	235	No	No	*Ile/Val and Val/Val*	[89]
Formaldehyde	172	No	No	No	[90]
Formaldehyde	151	No	No	No	[91]
Volatile organic compounds (toluene)	94	No	No	*Ile/Val and Val/Val*	[92]
Styrene	152	No	No	No	[93]
Antineoplastic drugs	104	No	No	No	[94]
Anaesthetic gases	99	*Null*	*Null*	NR	[95]
Pesticides	200	Equivocal[b]	Equivocal[b]	No	[96]
Pesticides	101	NR	NR	*Ile/Ile*	[97]
Pesticides	197	No	No	*Ile/Ile*	[98]
Pesticides	268	*Null*	No	NR	[99]
Pesticides	167	No	No	No	[100]
Pesticides	173	No	No	No	[101]
Pesticides	67	No	*Null*	NR	[102]

NR: not reported, PAH: polycyclic aromatic hydrocarbon.

[a] The genotype with high level of DNA strand breaks is described. For GSTM1 and GSTP1 they are null (deletion) or wildtype (positive). GSTP1 refers to the Ile105Val amino acid substitution, with valine type being less frequent than leucine. Ile/Val and Val/Val are sometimes pooled because of low number of subjects in the homologous group.

[b] Considered here to be equivocal because in one population ("rural") there was higher level of DNA strand breaks in subjects with GSTM1 null genotype, whereas GSTT1 genotype was not associated with DNA strand breaks. In another population ("urban) there was no gene-environment interaction with GSTM1, whereas GSTT1 null subjects the lower levels of DNA strand breaks as compared to subjects with wildtype genotype.

8. Other factors that may affect comet assay results

In addition to the factors described in the previous sections, there are other factors that may influence comet assay results such as variation in experimental procedures, infectious diseases, co-exposures and gene-environment interactions.

The comet assay is a relatively simple technique, but intra-laboratory variation is known to occur. The most important steps to keep stable are the final agarose content, lysis, alkaline treatment and electrophoresis [3,65–72]. Variation in staining and scoring of comets have not been investigated extensively, although differences in dyes and light intensity in fluorescence microscopes (aging of lamps) have been speculated to contribute to variation in comet assay results [3]. The effect of intra-laboratory variation depends on the way samples are analysed. Intra-laboratory variation is associated with attenuation of the exposure-effect relationship if samples are randomized before analysis. However, intra-laboratory variation has little effect in case samples are analysed as pairs in the same comet assay experiment (e.g. pre- and post-samples, or matched subjects from exposure and control groups). Intra-laboratory variation may produce an artificial association if there is uneven proportion of samples from exposed and controls in different comet assay experiments or if pre- and post- samples are analysed at different time points.

The effect of co-exposures should be considered in studies on environmental or occupational exposures. Examples of environmental exposures include metals, volatile organic compounds, pesticides and air pollution [21].

Infections – either acute or chronic – may give rise to higher levels of DNA damage or increased susceptibility to effect of DNA damaging agents (i.e. effect modifier). Effects of infections on comet assay results in humans have not been investigated in detail, although recent studies on COVID-19 patients indicate 3–5-fold higher DNA strand break levels compared to controls [73,74]. Nevertheless, infections as conditions are very diverse, covering different species of pathogens, target tissues and host immune responses.

Polymorphisms in genes encoding DNA repair proteins and xenobiotic metabolism may affect comet assay results through gene-environment interactions [75]. These are typically considered to be biomarkers of susceptibility or effect modifiers. As an example of gene-environment interactions, Table 4 summarizes results on effect modification by glutathione S-transferase (GST) polymorphism in studies with genotoxic effect by exposure to polycyclic aromatic hydrocarbons, fibres, formaldehyde, volatile organic compounds, antineoplastic drugs, anaesthetic gases and pesticides. GSTM1 (Gene ID: 2944), GSTP1 (Gene ID: 2950) and GSTT1 (Gene ID: 2952) have been assessed as effect modifiers in many studies because of their involvement of detoxification of hydrophobic and/or electrophile compounds. The polymorphisms of GSTM1 and GSTT1 are deletion, whereas the GSTP1 is an amino acid substitution in position 105 (isoleucine to valine). Overall, there are mixed results on GST polymorphism in terms of effect (9 out of 21 studies show effect modification) and direction of effect (both the most prevalent and rare polymorphism is reported to have highest effect). In general, the number of subjects is relatively small for assessment of gene-environment interaction of rare genotypes such as GSTP1 Ile105Val as the variant genotype is typically present in less than 10 % of the subjects. The same goes for some DNA repair genes such as OGG1 Ser326Cys polymorphism, which is typically present in approximately 5 % of the Caucasian population. In general, association between variation in xenobiotic metabolism and comet assay results should be handled as case-by-case approach, linked to specific environmental or occupational exposures for which a statistical interaction between exposure and gene polymorphism is justified by *a priori* hypothesis of effect modification.

9. Comments and perspectives

The previous sections have assessed various confounding factors that investigators should consider when designing a human biomonitoring study with analysis of DNA damage by the comet assay. Exhaustive physical activity and chronic diseases appear to be relatively strong

confounding factors. In fact, it is our impression that investigators carefully avoid the effect of these confounding factors in human biomonitoring studies, perhaps by excluding subjects with chronic diseases or exceptional conditions, such as running a marathon race before sampling. Variation over time (i.e. period or seasonal effect) seems to be a relatively strong contributor to variability in background levels of DNA strand breaks. Period effects have been a concern in antioxidant/phytochemical intervention trials, as it has been noted that sequential studies (i.e. samples obtained before and after supplementation) were more likely to report beneficial effects of supplementation on DNA damage levels than time-controlled designs such as crossover and parallel supplementation studies [76].

Common confounders (i.e. age, sex and smoking) have relatively weak and inconsistent effects in studies. These factors should not be neglected as confounders, but the modest effects provide some room for manoeuvring. Especially, restriction as a way of avoiding confounding by age, sex and even smoking may not be necessary. It opens the possibility for assessing effect modification. Other confounders, such as body weight (obesity, body mass index (BMI) or waist-hip ratio (WHR)), have not been sufficiently addressed in the studies included in the present review.

In general, potential bias related to effects of confounding factors is reduced in human biomonitoring studies with the comet assay by matching subjects. However, it is also clear that authors who have controlled for confounding by matching, do post-hoc statistical analyses in separate strata of confounders (e.g. men/women, old/young or smokers/non-smokers) to explore effect modification. This analysis is biologically sound, but there is also a worry that such analyses have been done post-hoc, and the statistical analysis should include a test of the interaction between the exposure and effect modifier (e.g. p-value of the interaction term in two-way ANOVA). In general, effect modification seems to be an effect that has been overlooked in human biomonitoring studies. Care should be taken when post-hoc statistical tests are done on conditioned groups because of concerns about collider bias.

It has not been the intention of the present review to provide a textbook introduction to various aspects of confounding. We recommend that epidemiologists and/or biostatisticians be involved in planning a human biomonitoring study. Taking the complexity of a human biomonitoring study into account, there are surprisingly many biomonitoring studies where comet assay results have been analysed by univariate tests, mainly Student's *t*-test (or Mann-Whitney *U* test) and Pearson correlation test (or Spearman correlation test). By using these tests, the major error is likely to be overestimation of effect size due to confounding and the multiple comparisons problem. It is advisable to consider multivariate tests, even in studies where confounders have been controlled by matching exposed subjects and controls. This is because the matching on confounding factors may be altered due to missing data in the statistical analysis (e.g. due to drop-outs, experimental failures or removal of outliers). Examples of multivariate tests can be found in studies on controlled exposure to wood smoke and diesel engine exhaust, where control for age and sex as confounders was done by study design (subjects acting as their own control) and statistical analysis due to missing data [77,78].

Unfortunately, there are no perfect rules regarding statistical analysis of datasets with covariates, mediators, modifiers, confounders and colliders. A general rule of thumb says that confounding should be considered when the effect size changes by more than 10 % from unadjusted to adjusted statistical analyses. For instance, if an exposure causes a net increase of 5 % of DNA in the comet tail in an unadjusted (univariate) test, confounding is likely if the effect is less than 4.5 % of DNA in the comet tail in multivariate tests, assuming that the covariate is a classic confounder. Again, it should be noted that adjustment for a mediator or collider may produce the same effect, and a changed effect size is not an indication of confounding. Fig. 2 depicts a flow diagram that may be of use for decisions on how to report effects of exposure in the comet assay. It is important to emphasise that the effect size, rather than the P-value, is the essential information. Depending on the type of relationship between the exposure and covariate(s), it will be most informative to report unadjusted and/or adjusted effect sizes. While investigators may want to dig into more advanced statistical models on comet assay results in human biomonitoring studies, it has to be cautioned that issues related to unintentional adjustment of mediators and collider adjustment bias may emerge. The way to avoid such flaws is careful consideration of causal pathways, assisted by direct acyclic graphs. This approach of drawing causal diagrams has been instrumental in epidemiology and forms the foundation of disentangling connections between exposures, other covariates and outcomes [79].

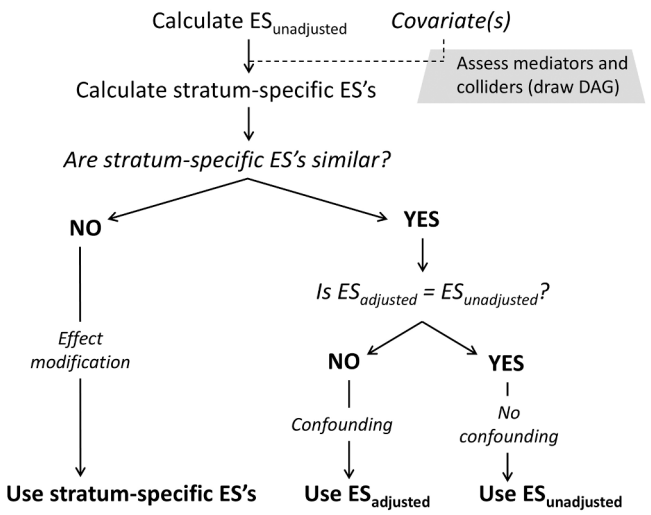

Fig. 2. Flow diagram for assessing confounding and effect modification by comparing unadjusted and adjusted effect size (ES) in statistical tests. Covariates include independent risk factors, confounders and effect modifiers. Mediators and colliders are assessed by, e.g. direct acyclic graphs (DAG) in the early step of the statistical analysis. (Various versions of the decision tree have been published in other articles and textbooks. It has not been possible to locate a source of the original version. In any case, the depicted decision tree is a modified version of these flow diagrams).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mrrev.2025.108566.

Data availability

Data will be made available on request.

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