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Inter-laboratory variation in measurement of DNA damage by the alkaline comet assay in the hCOMET ring trial

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Abstract

The comet assay is a simple and versatile method for measurement of DNA damage in eukaryotic cells. More specifically, the assay detects DNA migration from agarose gel-embedded nucleoids, which depends on assay conditions and the level of DNA damage. Certain steps in the comet assay procedure have substantial impact on the magnitude of DNA migration (e.g. electric potential and time of electrophoresis). Inter-laboratory variation in DNA migration levels occurs because there is no agreement on optimal assay conditions or suitable assay controls. The purpose of the hCOMET ring trial was to test potassium bromate ($KBrO_3$) as a positive control for the formamidopyrimidine DNA glycosylase (Fpg)-modified comet assay. To this end, participating laboratories used semi-standardized protocols for cell culture (i.e. cell culture, $KBrO_3$ exposure, and cryopreservation of cells) and comet assay procedures, whereas the data acquisition was not standardized (i.e. staining of comets and image analysis). Segregation of the total variation into partial standard deviation (SD) in % Tail DNA units indicates the importance of cell culture procedures (SD = 10.9), comet assay procedures (SD = 12.3), staining (SD = 7.9) and image analysis (SD = 0.5) on the overall inter-laboratory variation of DNA migration (SD = 18.2). Future studies should assess sources of variation in each of these steps. On the positive side, the hCOMET ring trial demonstrates that $KBrO_3$ is a robust positive control for the Fpg-modified comet assay. In conclusion, the hCOMET ring trial has demonstrated a high reproducibility of detecting genotoxic effects by the comet assay, but inter-laboratory variation of DNA migration levels is a concern.

Keywords: Comet assay; DNA damage; inter-laboratory variation; ring trial; validation

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Editor's choice

Introduction

The alkaline comet assay is a widely used technique to measure DNA strand breaks and oxidatively damaged DNA in eukaryotic cells by assessment of DNA migration in agarose gels after electrophoresis. The level of DNA migration depends on the number of lesions and assay conditions. In theory, if assay conditions are identical, the DNA damage level is proportional to DNA migration level, but in practice differences in assay conditions affect the obtained results and they are important factors to consider, when comparing results from different laboratories [1]. It might seem straightforward to develop standard operating procedures to reduce inter-laboratory variation. However, such approaches have not proven successful so far, when it comes to reducing inter-laboratory variation in DNA migration levels.

The standard protocol of the alkaline comet assay measures DNA strand breaks and alkali-labile sites. A modified version of the assay uses DNA repair enzymes to create DNA strands at specific lesions, which are stable in the standard alkaline condition of the assay. By using formamidopyrimidine DNA glycosylase (Fpg), it is possible to detect oxidatively damaged DNA. Twenty years ago, the Fpg-modified comet assay was used in a project called the European Standards Committee on Oxidative DNA damage (ESCODD) to gain knowledge on the background level of oxidatively damaged DNA in mammalian cells [2,3]. The ESCODD ring trial revealed a substantial inter-laboratory variation in levels of Fpg-sensitive sites when laboratories analysed batches of the same cells [2,3]. Moreover, it was demonstrated that 'biological variation' in levels of Fpg-sensitive sites in peripheral blood mononuclear cells, which were isolated and analysed in different laboratories, was mainly due to inter-laboratory differences in assay conditions [4]. Since the ESCODD project, efforts have been made to understand and reduce the variation in DNA migration levels within or among laboratories [5–17]. The strongest approaches have been multi-laboratory projects of which three have included ring trails on DNA damage measurements by the comet assay, namely the *European Comet Assay Validation Group* (ECVAG) [10,18–23], *Comet assay and cell array for fast and efficient genotoxicity testing* (COMICS) [17], and *The comet assay as a human biomonitoring tool* (hCOMET) [24].

In order to mitigate problems related to inter-laboratory variation in DNA damage and repair activity in human samples, hCOMET partners have published a number of articles with the aim of describing and reducing variation in comet assay procedures and results, including a review on technical aspects of steps in the comet assay procedure that are known to have strong influence on the level of DNA migration [1], procedures for optimal cryopreservation of blood cells [25], detailed assay protocols [26,27], and recommendations on essential information in published papers on the comet assay [28]. Two comprehensive reviews have discussed the use of the comet assay in biomonitoring studies, including issues related to confounding factors, study design and statistical analysis of data [29,30]. Lastly, hCOMET partners have collected individual comet assay results from human population studies, and pooled analysis of these data showed a large inter-laboratory variation in DNA strand break values [31].

Alongside these works, hCOMET partners carried out a ring trial to assess potassium bromate ($KBrO_3$) as a positive control for the Fpg-modified comet assay [24]. The first experiments demonstrated a high reproducibility of the generation of Fpg-sensitive sites in monocytic THP-1 cells in different laboratories [24]. A subsequent study extended this work by showing that 3 years of cryopreservation at –80°C did not affect the level of DNA strand breaks and Fpg-sensitive sites in the samples [32,33]. Despite these positive findings, there has been a large difference in levels of DNA migration among laboratories. In an attempt to assess whether the inter-laboratory variation stems from differences in the measurement of DNA migration, hCOMET partners have assessed visual scoring of comets as an alternative to image analysis [34].

The present paper provides an integrated analysis of the hCOMET ring trial results. Moreover, many steps in the comet assay procedure are considered to influence the DNA migration level and are therefore worthwhile to be standardized to reduce inter-assay and inter-laboratory variation. However, there are steps in the procedure where empirical evidence has been lacking because they are not usually considered to be important factors for the variation in the comet assay (e.g. staining and scoring). Based on the results in the hCOMET ring trial, the present paper assesses the magnitude and sources of inter-laboratory variation in comet assay results.

Method

The present study is an integrated analysis of results from the hCOMET ring trial, which are previously reported in four different papers [24,32–34]. The earlier publications addressed specific research questions, whereas the present paper integrates the results in an overall analysis of the inter-laboratory variation in the comet assay. In addition, the paper contains previously unpublished results on inter-laboratory variation in scoring of digitalized comet images.

In this paper, we describe comets in terms of *DNA migration* measured either by image analysis (i.e. %Tail DNA) or by visual scoring (i.e. arbitrary units). We have used a 5-class visual scoring system, which was assessed in a separate arm of the ring trial [34]. *DNA strand breaks* and *Fpg treatment* refer to comet assay experiments where lysed nucleoids have been incubated with buffer or Fpg, respectively. In certain analyses, we have calculated the net difference in DNA migration owing to the Fpg treatment (i.e. *Fpg-sensitive sites* = *Fpg - buffer*). $KBrO_3$ has been used as genotoxic agent; it generates Fpg-sensitive sites with little concurrent increase in DNA strand breaks [35].

We have structured the discussion of comet assay variation in a 'reverse' manner, starting with the analysis of DNA migration in comets and subsequently moving backwards in the comet assay procedure. The comet assay experiments were done according to a semi-standardized procedure where the percentage of low melting point agarose (0.7% final concentration), lysis time (1 h), Fpg treatment (1 h), alkaline unwinding (20 min), and electric potential and time of electrophoresis (20 V/cm × min) were standardized. In addition, cell culture procedures, $KBrO_3$ treatment and cryopreservation were standardized and all laboratories used Fpg from the same source (NorGenotech, Oslo, Norway). All laboratories used THP-1 cells from the same batch of cells. Figure 1 depicts the hCOMET experiments, which have been segregated into cell culture procedures, comet assay procedure, and data

acquisition. The present paper addresses mainly the late stage of the experimental protocol because other steps were standardized.

Statistical analysis

We use standard deviation (SD) as descriptor of the variation in DNA migration. The SD has the advantage of being the same unit as the mean, but there are also some critical issues that should to be taken into account when comparing SDs from different studies. The SD decreases with increasing data number in the computation, which means that comparison of datasets with different number of results is slightly biased towards lower variation in dataset with results from many laboratories. On the other hand, there is a tendency for the SD to increase with the level of DNA migration [34]. Lastly, SDs of independent factors cannot be added, whereas variances can. We have used coefficient of variation (CV) in analyses based on direct comparison of primary comet descriptors on the same dataset. As the CV is a ratio between SD and mean, it has the same critical issues as the SD and should be interpreted with caution when different datasets are compared.

For statistical analysis of the results, we have used regular or mixed model linear regression with *laboratory*, *study* or *mean/median* comet distribution descriptor as categorical (absorbed) factor. Slopes of the regression models are reported with their corresponding standard error of the mean (SEM). In addition, we report 95% confidence interval (95% CI) for slopes or effects. Groups with non-overlapping 95% CI are statistically significant at $\alpha = 0.05$ level. The statistical analysis was carried out in Stata 15 (Stata Corp, College Station, TX, USA).

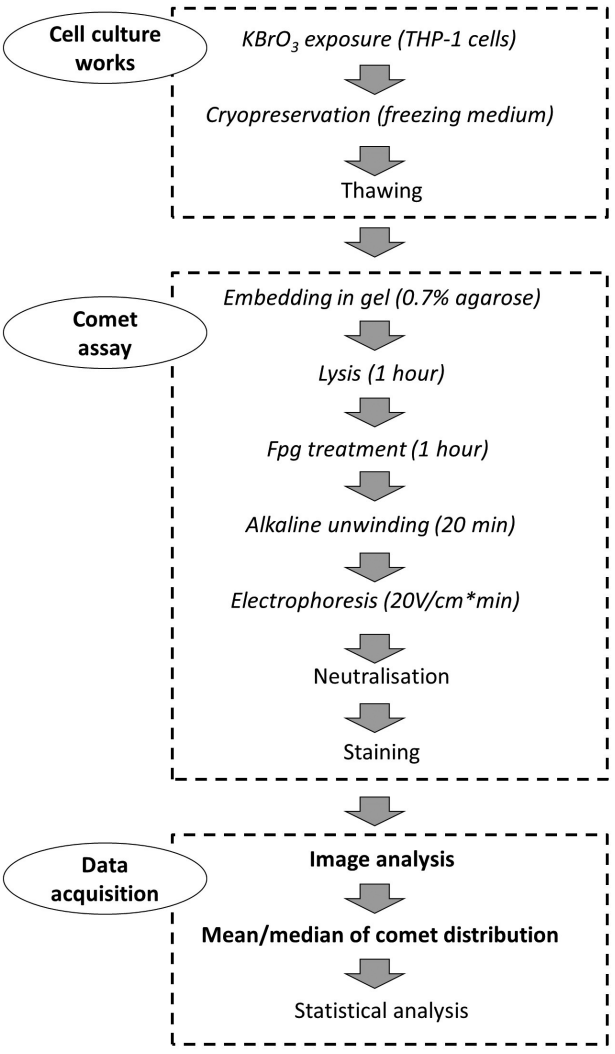

Figure 1. Flow of cell culture procedures (procuring of samples), comet assay procedure and data acquisition. Steps with italic text are standardized in the hCOMET ring trial. Research questions in the present paper pertain to the data acquisition step (bold text).

Results and discussion

Inter-laboratory variation of DNA strand breaks and Fpg-sensitive sites in human blood cells

The hCOMET ring trial addresses the magnitude of variation in DNA migration values. In order to have a suitable reference point, it is worthwhile drawing attention to the variation in levels of DNA migration from published biomonitoring studies. Figure 2 provides a summary of results from different studies on human leukocytes, including the hCOMET cohort [31] and reviews on DNA damage levels in control groups in biomonitoring studies [35,36]. Assessed as %Tail DNA, the mean and SD of DNA strand breaks and Fpg-sensitive sites are 9.6 ± 5.9 and 6.7 ± 4.7 in studies before 2006, respectively [36]. More recent studies indicate a larger variation (DNA strand breaks: 7.0 ± 7.0, Fpg-sensitive sites: 9.6 ± 9.5) [31,35]. The larger inter-laboratory variation in recent years might be because there are more laboratories using the comet assay and therefore also potentially more variation in laboratory procedures. Regardless of the sources of inter-laboratory variation, the SD is approximately 7–10% Tail DNA in leukocytes from healthy humans with relatively low levels of DNA damage.

Difference between means and medians of the comet distribution

A persistent discussion among researchers who use the comet assay is whether the mean or median is the best way to describe the central estimate of the comet distribution [37]. The distribution of comets is rarely a normal distribution, which would dictate the use of median or other descriptors of a non-normal distribution. The median is very easy to calculate and therefore the preferred descriptor among researchers who do not prefer the mean. More advanced descriptors of the comet distribution such as the Chi-square or Weibull models have been described, but they are not used because they are difficult to compute [37]. Thus, the most relevant question is whether the observed inter-laboratory variation in DNA migration values can be attributed to the non-standardized use of either mean or median between laboratories.

We have compared the relationship between $KBrO_3$ exposure and DNA migration values by using either mean or median of the comet distribution. In these statistical analyses, the $KBrO_3$ exposure is the predictor of genotoxic effect. The results are analysed by linear mixed model with the $KBrO_3$ concentration as continuous factor and median/mean as categorical (absorbed) factor. In addition, we have analysed the inter-laboratory variation in separate strata of mean and median results, using linear regression with $KBrO_3$ concentration

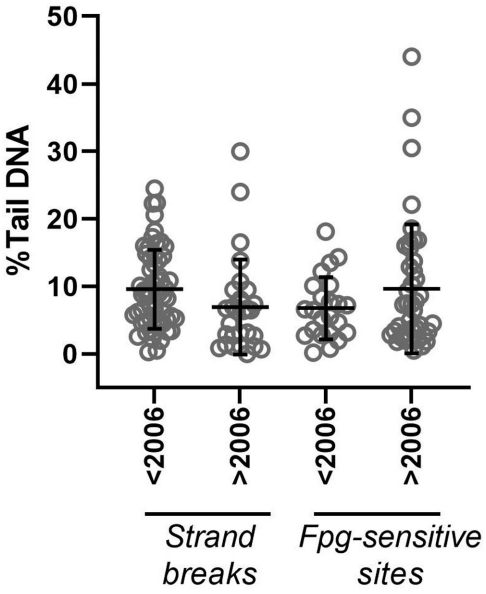

Figure 2. Inter-laboratory variation in DNA strand breaks and Fpg-sensitive sites in human leukocytes. The results have been compiled from reviews on biomonitoring studies that have been published before 2006 (<2006) [36], or hCOMET cohort [31] and biomonitoring studies from 2006 to 2018 (>2006) [35]. Each grey symbol is the mean DNA migration value in cells from subjects of the control group in the biomonitoring study. The mean and standard deviation are shown as lines and error bars.

as continuous factor. Table 1 shows that the genotoxic effect is not affected by using mean or median (i.e. same slopes and R^2-values by the statistical models of datasets on DNA strand breaks, Fpg treatment and Fpg-sensitive sites). However, there is a slightly higher intercept in the statistical analysis of DNA strand breaks by using the mean as compared to the median (i.e. non-overlapping 95% confidence interval of the models *SB (mean)* and *SB (median)*, or 2.08% Tail DNA higher value by using the mean as compared to median, $P < 0.001$). Table 2 shows that DNA strand break values are consistently higher when obtained as the mean of the comet distribution as compared to the median. Slightly higher intercept values are also obtained in samples treated with Fpg (i.e. 1.51% Tail DNA in Table 1), although there is more experimental variation as reflected in a wider 95% confidence interval. The smaller differences between mean and median values in the datasets on Fpg-treatment and Fpg-sensitive sites might be due to a larger inter-laboratory variation of the Fpg incubation step (e.g. slight differences in temperature of the incubation or moisture in the incubation units), which blurs the small difference related to using mean or median comet distribution descriptors.

It can be speculated that a mixed use of mean and median values may contribute to inter-laboratory variation in DNA damage [38]. Figure 3A shows results from an analysis to assess the impact of the difference between using the mean or median as comet distribution descriptor for DNA strand breaks. Using mean and median of the comet distribution produces similar inter-laboratory variation (SD = 2.9% or 2.7% Tail DNA, respectively), which is not surprising because the mean and median are highly correlated ($r = 0.95$, $P < 0.001$; Fig. 3B). Results on DNA strand breaks from the four $KBrO_3$ exposure groups are pooled because there is no concentration–response relationship in strata of the individual laboratories. Furthermore, we have assessed inter-laboratory variations by random selection of mean or median values from datasets in each laboratory and run 1000 simulations to illustrate the spread in the inter-laboratory variation. As it can be seen in Fig. 3A, random selection of means or medians produces datasets that have either lower or higher inter-laboratory variation. The lower, mean and upper boundaries of inter-laboratory SD are 1.8%, 2.9%, and 3.8% Tail DNA, respectively. Figure 4 depicts the same analysis of results and simulation for Fpg-treatment of $KBrO_3$ exposed cells, stratified into different exposure groups because there is a difference in levels of oxidatively damaged DNA. The SD of results on medians is higher than the SD of means, and random selection of the mean or median has little effect on the inter-laboratory variation. Importantly, it indicates that non-standardized use of mean or median values of the comet distribution is not a systematic factor for inter-laboratory variation in DNA migration values.

Variation in analysis of digitalized comet images

In the hCOMET ring trial, we tested the variation in analysis of the same digitalized comet images. All laboratories except two use software from the same company (i.e. Comet IV from Perceptive Instrument, nowadays Instem; the image analysis system is referred to as 'Perceptive' in the present paper). Ten files of comet images with 33 highlighted comets were distributed to hCOMET partners. The comet image files are available as supplementary information (called 'Slide 1.bmp' to 'Slide 10.bmp'), including a guide to locate the specific comets (Supplementary Document 1) and raw data of the experiment (Supplementary Document 2). DNA migration was first measured on ten different days by the same investigator in a central laboratory to ascertain the measurability of the comets and to obtain information on intra-laboratory variation in image analysis of comets. Nine other laboratories analysed the comets by image analysis too. Some laboratories scored the comets two to three times due to difficulty in image analysis or discrepant results. The experiment was designed to unravel variation in image analysis of individual comets and the central estimate of all comets in the sample. In addition, the dataset covers a very broad range of comets to assess if variation in image analysis is related to the level of DNA migration. Results from two laboratories were re-assessed because of a relatively high background level of DNA migration in the first analyses (laboratory 2 and 7). Laboratory 7 readjusted settings in the software system and obtained results that were similar to other laboratories, although still slightly higher. Laboratory 2 had mainly used an interactive/manual tool in the software to set boundaries of comet heads and tails. Another laboratory analysed comets with either interactive/manual analysis or automatic analysis (laboratory 3). Measurement of comets with the interactive tool led to higher values of DNA migration than automatic analysis of the same comets in laboratory 3. Although the original measurements produced a higher level of DNA migration, there are excellent correlations with DNA migration values in laboratories that scored the same comets ten times (i.e. correlation coefficients of 0.98, 0.96, and 0.93 in laboratory 2, 3, and 7, respectively).

Figure 5A and B show the variation in analysis of individual comets by the same laboratory or different laboratories. As can be seen, there does not appear to be a relationship be-

Table 1. Statistical analysis of DNA migration in the comet assay of monocytic THP-1 cells that have been exposed to $KBrO_3$.

Dependent factor[a]	Comet distribution descriptor[b]	$KBrO_3$ concentration (slope)	Intercept	R^2_{model}
SB (overall)	2.08 (1.40, 2.77)***	0.29 (0.10, 0.49)**	3.01 (2.43, 3.59)	0.46
SB (mean)	Not applicable	0.27 (–0.03, 0.57)	5.13 (4.42, 5.85)#	0.41
SB (median)	Not applicable	0.32 (0.06, 0.57)*	2.97 (2.35, 3.59)	0.46
Fpg (overall)	1.51 (–1.12, 4.13)	6.97 (6.22, 7.72)***	8.53 (6.31, 10.8)	0.71
Fpg (mean)	Not applicable	6.73 (5.68, 7.79)***	10.4 (7.91, 12.9)	0.70
Fpg (median)	Not applicable	7.21 (6.10, 8.32)***	8.15 (5.50, 10.8)	0.71
Fpg-sensitive sites (overall)	–0.48 (-3.00, 2.03)	6.89 (6.17, 7.61)***	5.94 (3.81, 8.07)	0.76
Fpg-sensitive sites (mean)	Not applicable	6.67 (5.67, 7.68)***	5.80 (3.41, 8.19)	0.72
Fpg-sensitive sites (median)	Not applicable	7.10 (6.02, 8.17)***	5.59 (3.03. 8.15)	0.72

The statistical analysis is based on all data ('overall', including both mean and median values) or separate strata of 'mean' or 'median' values. Results in brackets are 95% CI.

aSB is the standard comet assay (nuclei treated with buffer), Fpg refers to the level of DNA migration in samples treated with Fpg (i.e. sum of DNA strand breaks and Fpg-sensitive sites), and Fpg-sensitive sites are the difference between nuclei treated with Fpg and buffer.

bSingle-factor effect of mean (i.e. median is baseline). The statistical model assumes a similar concentration-response relationship between $KBrO_3$ exposure and DNA migration in datasets on mean or median comet distribution descriptor.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ statistically significant effect of $KBrO_3$ exposure on DNA migration. #$P < 0.05$, statistically significant from the intercept of the median values.

Table 2. DNA migration levels of DNA strand breaks by using mean or median as comet distribution descriptor[a].

Laboratory	Mean[b]	Median[b]	Delta-value[c]
1	10.2 ± 4.84 ($P = 0.23$)	6.59 ± 3.66	3.64 ± 2.75***
2	2.13 ± 2.56 ($P = 0.45$)	0.75 ± 2.15	1.37 ± 0.90***
3	3.76 ± 3.08 ($P < 0.05$)	1.29 ± 1.45	2.47 ± 1.68***
4	3.77 ± 2.24 ($P = 0.12$)	2.15 ± 1.56	1.62 ± 0.94***
6	5.00 ± 2.69 ($P = 0.68$)	3.26 ± 2.96	1.74 ± 0.77***
7	8.74 ± 4.96 ($P = 0.38$)	8.16 ± 4.04	0.58 ± 1.39 (P=0.08)
8	3.54 ± 1.95 ($P < 0.001$)	1.02 ± 1.53	2.51 ± 1.42***
9	3.53 ± 1.48 ($P = 0.24$)	1.46 ± 1.13	2.07 ± 0.53***
10	8.28 ± 5.30 ($P = 0.74$)	5.30 ± 5.08	2.98 ± 1.01***

aThe data (%Tail DNA) are *mean of means* or *mean of medians* of samples from all experiments, including different concentrations of $KBrO_3$.

bThe results are means and standard deviation (*P*-value of *F*-test for difference in distribution between results in datasets on means and medians).

cThe results are mean and standard deviations of delta values of paired mean and median values (delta value = mean – median) and paired-sample Student's *t*-test. *$P < 0.05$, ***$P < 0.001$.

tween the level of DNA migration and variation in day-to-day image analysis by the same investigator (Fig. 5A) or investigators in different laboratories (Fig. 5B). In addition, it is not the same comets that display variation when measured repeatedly in the same laboratory and measured once in different laboratories (Fig. 5C). Despite variation in measurement of certain individual comets, there is a very high consistency in mean values of the comet distribution within the same laboratory and between laboratories (Fig. 5D).

Relationship between comet shape and %Tail DNA measured by image analysis

An important question is whether investigators exert censorship when analysing comets by image analysis systems. Investigators may interpret DNA migration in comets differently, which may lead to inter-investigator variation in image analysis if there are subjective criteria for accepting values of primary comet descriptors. This was assessed in the hCOMET ring trial in an experiment where slides were produced in a central laboratory and then shipped to other laboratories where they were stained and scored by image analysis or visual scoring [34]. Monocytic THP-1 cells were exposed to different concentrations of H_2O_2 (0, 10, 25, or 50 µM for 5 min) to generate a difference in DNA migration (Fig. 6). As can be seen in Fig. 6A, there is not a one-to-one relationship between values of visual score and %Tail DNA. However, the inter-laboratory CV is similar regardless of comets having been scored by image analysis or visual scoring (Fig. 6B). Figure 7 depicts the relationship between visual scoring and image analysis at individual investigator level. The DNA migration values are normalized to Z-scores within each exposure group (i.e. 0, 10, 25, and 50 µM H_2O_2). As can be seen, there is a weak association between the Z-score of visual scoring and image analysis ($r = 0.38$, $P < 0.05$, linear regression), suggesting that a part of the inter-investigator variation in %Tail DNA is due to a difference in the shape of comets.

Visual scoring has been used concurrently with software-assisted image analysis to measure DNA migration in the comet assay. There are visual scoring systems with different numbers of classes of comets, but the five-class system has been most popular. The five-class system was introduced in early the 1990s [39]. It has remained unchanged since the introduction; *Class 0* comets are round and *class 4* comets have heads with reduced size (compared with the class 0

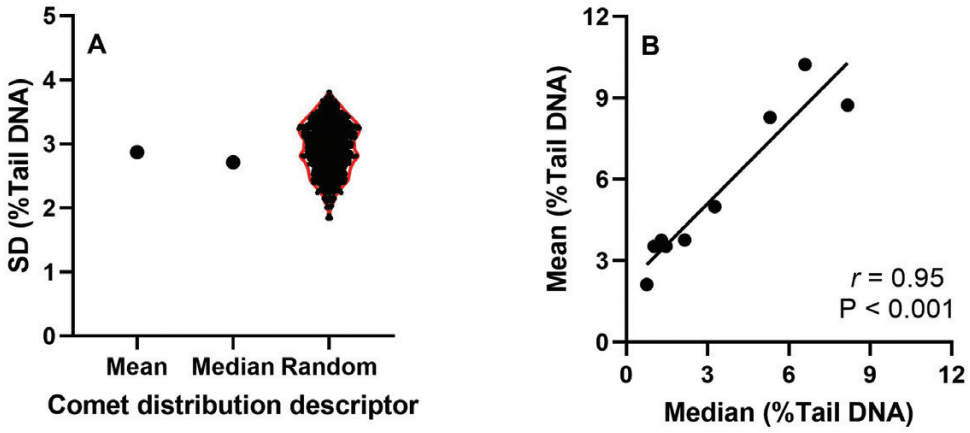

Figure 3. Inter-laboratory variation in DNA strand breaks related to use of mean or median of comet distribution descriptor. (A) The symbols are the standard deviation (SD) of DNA migration values from datasets in nine different laboratories. Black circles are inter-laboratory variation of means (SD = 2.9% Tail DNA, coefficient of the mean = 53%, n = 9) or medians (SD = 2.7% Tail DNA, coefficient of the mean = 81%, n = 9). A simulation of inter-laboratory variation from random use of mean or median comet distribution is depicted as probability density (violin) plot (generated in GraphPad Prism 9). Medium smoothing of the density curve has been used to show the shape of the violin plot. The plot is based on 1000 simulations and random selection of mean or median from each laboratory (random generator tool in Excel, SD = 2.9% Tail DNA, min = 1.8% Tail DNA, max = 3.8% Tail DNA). (B) Correlation between mean and median results. Symbols are individual laboratories.

comet) and show less fluorescent intensity, while most of the DNA is in the tail. Figure 8 depicts results from studies that have assessed the relationship between levels of visual score and %Tail DNA [16,18,39–45]. The studies have been published over a period from 1995 to 2022, but they can somewhat arbitrarily be segregated into 'early' (1995–2003) [39–43] and 'late' (2010–2022) studies [16,18,34,44,45]. In general, there is high correlation between comets scored by visual scoring and image analysis (r-values of 0.99 and 0.98 in datasets of early and late studies, respectively). However, the slope in the early studies (0.78 ± 0.03, 95% CI: 0.72, 0.84) is larger than the slope in the late studies (0.58 ± 0.03, 95% CI: 0.51, 0.64). The difference in slopes over time raises a question of whether different software systems give rise to variation the measurement of DNA migration. Image analysis in the earlier studies was done by using mainly a software system called Kinetics, whereas the Perceptive software is the most used in the laboratories of hCOMET ring trial participants. However, analysis of this difference in results from different image analysis software in the dataset from the ECVAG ring trial does not indicate such an effect. Figure 9 depicts the results from an analysis where premade slides were distributed to different laboratories that stained and scored the samples by image analysis systems from either Perspective or Kinetics/other systems. The results do not indicate that using image analysis systems from Perceptive as compared to other systems produces a systematic variation in DNA migration levels from different laboratories. This is in agreement with another study that concluded %Tail DNA values of comets with different stains and image analysis systems were not substantially different [46].

Sources of inter-laboratory variation in DNA migration

The analysis of samples in the hCOMET ring trial captures the variation of different steps of cell culture and comet assay procedures (Fig. 1). For simplicity, the samples are called *Comet images*, *Premade slides*, *Central scoring (1)* slides, *Central scoring (2)* comet images, *Ro19-8022* and *Own analysis*. The samples have been produced and/or analysed in different laboratories, which makes it possible to estimate the contribution of different sources of variability to the inter-laboratory variation in comet assay results. Figure 10 shows the exchange of samples between individual laboratories and the two central laboratories. Table 3 outlines the different parts of the procedure each sample covers (i.e. pluses in the table). Unfortunately, the variation in DNA migration values depends on the level of DNA migration. Figure 11 shows this relationship between mean levels of DNA migration and SD in different steps of the comet assay. Thus, it is necessary to use a fixed level of DNA migration to assess the inter-laboratory variation in results. The estimates of the inter-laboratory variation in Table 3 are based on the SD at a fixed level of 25% Tail DNA. This value has been chosen because it is in the middle of the range of values and included in all regression lines in Fig. 11.

There is a relatively good relationship between the number of steps the sample covers (i.e. number of plusses) and the magnitude of variation at 25% Tail DNA (last row in Table 3). A description of the samples and their use as estimates of variation are as follows:

Comet images: the same comet images were analysed by all laboratories (previously unpublished results, Fig. 5). Thus, these samples describe the variation in image analysis. As expected, there is relatively little variation between laboratories when means of the DNA migration of digitalized comet images are analysed (SD = 0.5) (Fig. 11).

Premade slides: slides with comets from H_2O_2-exposed cells were produced in one laboratory and shipped to other laboratories. Thus, the variation in DNA migration originates from staining and scoring (Fig. 6) [34]. As can be seen in Fig. 11, there is more variation in the premade slides than comet images (SD = 7.9 versus SD = 0.5 at 25% Tail DNA), demonstrating the strong contribution of differences in staining to the inter-laboratory variation.

Central scoring (1): The analysis was carried out in the first round of experiments in the ring trial [24]. Individual laboratories analysed their own samples of $KBrO_3$ exposed cells by

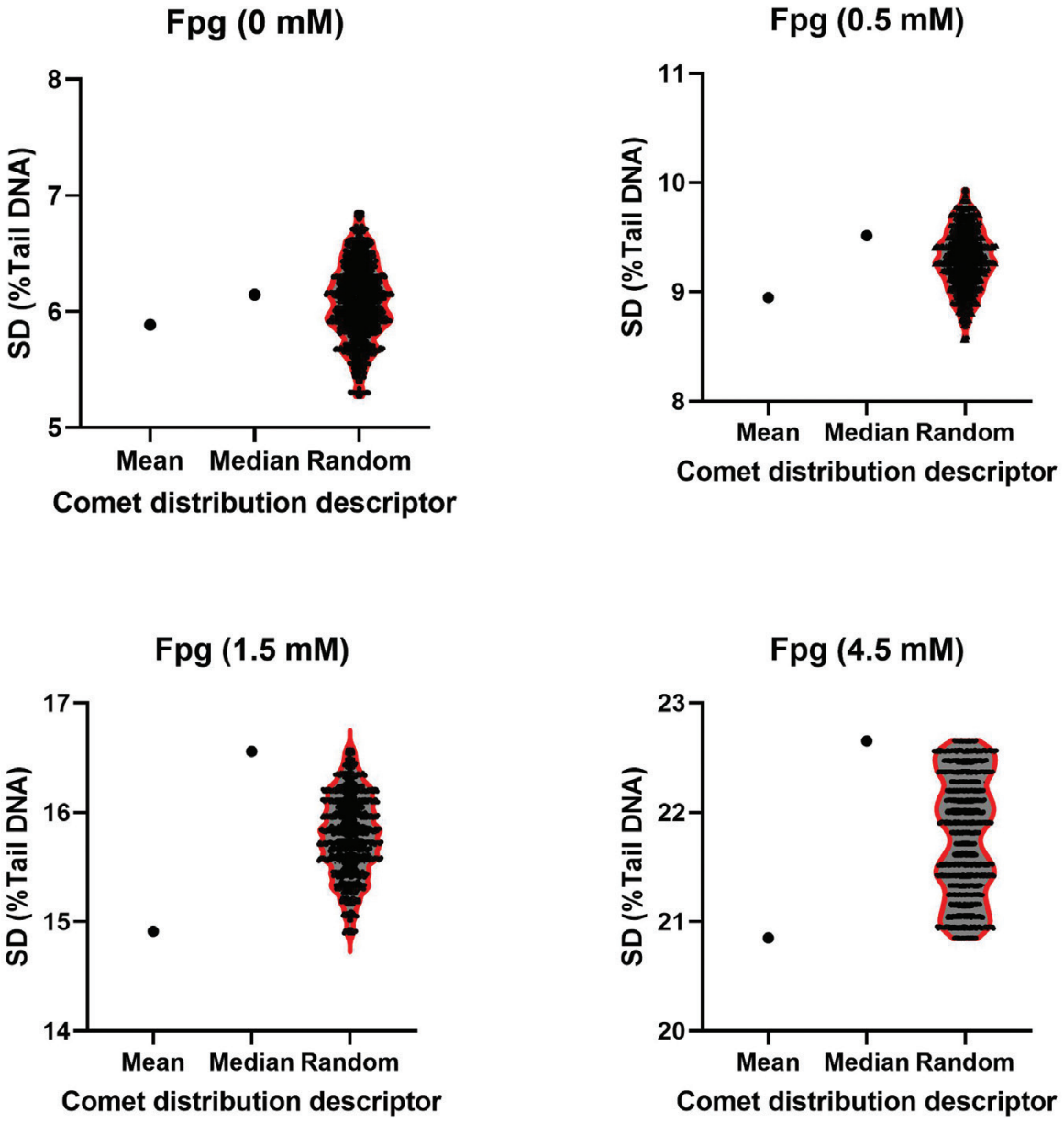

Figure 4. Inter-laboratory variation in DNA migration related to use of mean or median of comet distribution descriptor on samples of $KBrO_3$ exposed cells and incubation with Fpg in the comet assay analysis. The symbols are the standard deviation (SD) of DNA migration values from datasets in eight different laboratories. Simulations of inter-laboratory variation from random selection of mean or median of the comet distribution is depicted as probability density (violin) plots (generated in GraphPad Prism 9). Medium smoothing of density curve has been used to show the shape of the violin plot. The plots are based on 1000 simulations and random selection of mean or median from each laboratory (random generator tool in Excel).

the comet assay and forwarded unstained slides to a central laboratory, which scored all samples by the same image analysis system. Thus, the variation originates from heterogeneity in cell culture works and other comet assay steps than staining and scoring of comets. As can be seen in Fig. 11, there is more variation in samples that were scored in the central laboratory than premade slides (SD = 11.3 versus 7.9 at 25% Tail DNA), indicating a stronger contribution of differences in cell culture and comet assay procedures than staining and image analysis to the overall inter-laboratory variation.

Central scoring (2): The analysis was carried out in the second or third round of experiments in the ring trial [32]. Individual laboratories analysed their own samples of $KBrO_3$ exposed cells by the comet assay and forwarded digitalized images to a central laboratory, which scored all samples by visual scoring (five-class system). These scores have then been converted to %Tail DNA by using an investigator-specific standardization curve where 1 a.u. corresponds to 0.48% Tail DNA [16]. The variation in these samples originates from heterogeneity in cell culture procedures and other comet assay steps than scoring of comets. As can be seen in Fig. 11, there is slightly higher variation in the *Central scoring (2)* sample set than *Central scoring (1)* (SD = 12.1 versus 11.3 at 25% Tail DNA). The difference might be due to variation in staining procedures or to the fact that the experiments in *Central scoring (1)* and *Central scoring (2)* were not done at the same time (i.e. variation from experiment to experiment).

Ro19-8022: samples of cryopreserved Ro19-8022 exposed cells were obtained in a central laboratory and shipped to

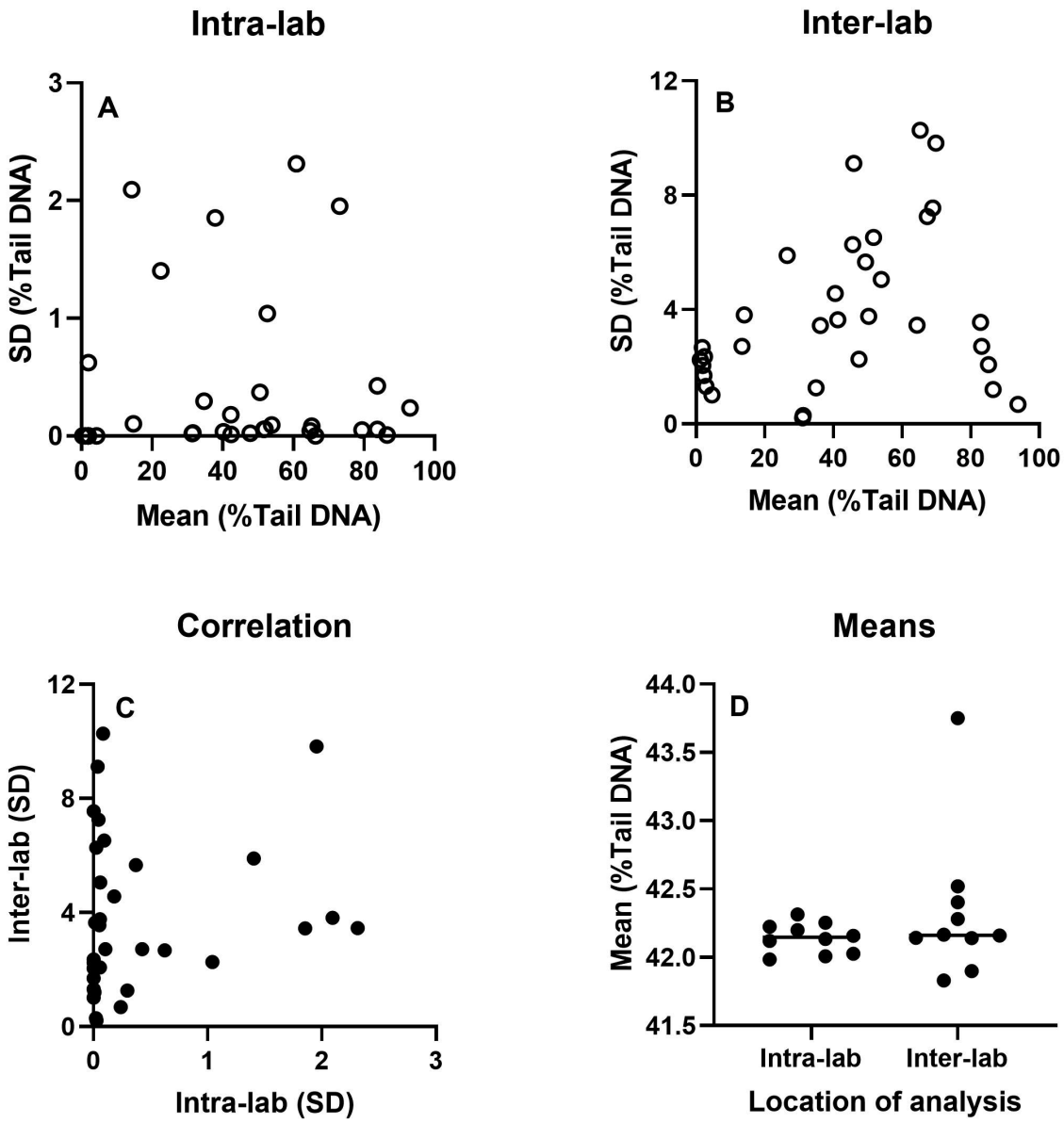

Figure 5. Mean and standard deviation of DNA migration in comets. Ten laboratories scored the same digitalized images. (A) and (B) display the intra-laboratory and inter-laboratory standard variation (SD) in 33 different comets with different level of DNA migration, respectively. (C) Shows the correlation between the variation in %Tail DNA values of comets measured 10 times in a single laboratory or in 10 different laboratories. Each symbol in panel A, B, and C is one comet. (D) Shows the mean of the comet distribution in ten measurements in a single laboratory or one measurement in 10 different laboratories. The symbols in panel D are the mean level of DNA migration of 33 comets, which were scored 10 times by the same investigator ('intra-lab') or once by 10 different investigators ('inter-lab').

other laboratories as reference samples [24]. The analysis is based on results in the first round of experiments because it includes the highest number of laboratories. The inter-laboratory variation in these samples covers all steps except the cell culture procedures. As can be seen in Fig. 11, there is more variation in *Ro19-8022* samples (SD = 14.6 at 25% Tail DNA) than in the *Premade slides*, *Central scoring (1)* and *Central scoring (2)* samples.

Own analysis: this covers the variation in the entire experiment. The estimate of SD is based on results from the first round of experiments because the majority of laboratories assessed both DNA strand breaks and Fpg-sensitive sites in the early part of the ring trial. As can be seen in Fig. 11, these samples have the highest level of inter-laboratory variation (SD = 18.2 at 25% Tail DNA).

Based on the segregation of the experiments into steps of the procedure, it is possible to estimate the partial SD for each of the steps (Table 3). This indicates a ranking of sources of the inter-laboratory variations as follows: comet assay procedure (SD = 12.3), cell culture procedures (SD = 10.9), staining (SD = 7.9), and image analysis (SD = 0.5). An interesting aspect of the analysis is the high inter-laboratory variation in the premade slides, which indicates that a relatively large contributor to the overall inter-laboratory variation is differences in staining and scoring (SD = 7.9). Thus, staining and scoring might be easy to standardize steps in the comet assay procedure to mitigate inter-laboratory variation in DNA migration values. We have assessed the relationship between differences in staining and image analysis by comparing the variation in results in premade slides and

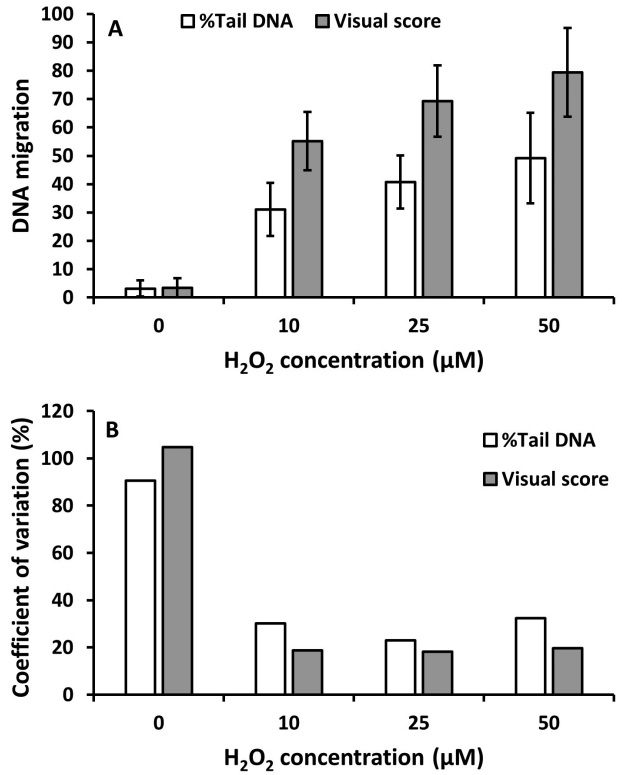

Figure 6. Analysis of DNA migration by image analysis (white bars) and visual scoring (grey bars) of comets. The gradient in DNA migration has been created by exposing monocytic THP-1 cells to hydrogen peroxide (H_2O_2). Each laboratory stained and scored comets in premade slides (Gelbonds). The results are reported as mean and standard deviation (A) and coefficient of variation (B). The results are based on measurements in ten laboratories.

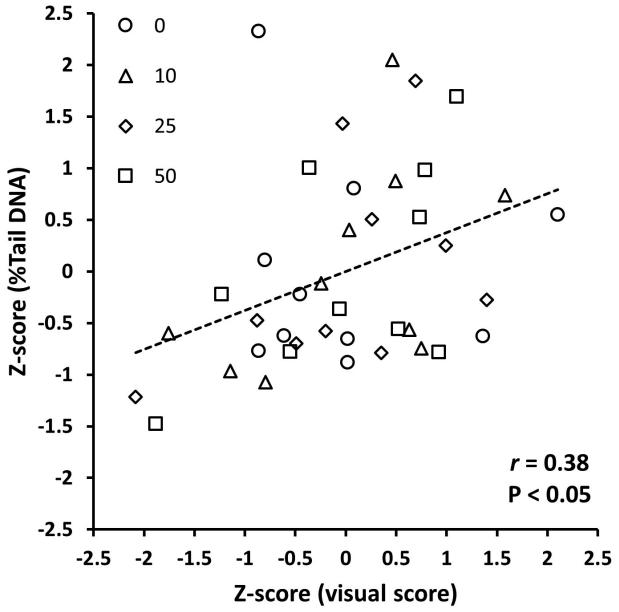

Figure 7. Correlation between visual scoring and image analysis of premade slides of THP-1 cells that have been exposed to hydrogen peroxide (H_2O_2) and processed in a central laboratory before shipping to other laboratories for staining and scoring by visual scoring (five-class system) and image analysis (%Tail DNA). The DNA migration values are normalized to Z-scores within each exposure group (i.e. 0, 10, 25, and 50 μM H_2O_2).

the levels of DNA strand breaks and Fpg-sensitive sites in $KBrO_3$-exposed THP-1 cells from the same laboratory (Fig. 12). The analysis is based on standardized results (Z-score) of both premade slides and $KBrO_3$ exposed cells. There are weak positive correlations between levels of DNA migration in premade slides and DNA strand breaks ($r = 0.64$, $P < 0.05$) and Fpg-sensitive sites ($r = 0.28$, not statistically significant) in $KBrO_3$ exposed THP-1 cells. It is possible that the weaker correlation of the Fpg-sensitive sites is due to variation in the Fpg treatment. Thus, a simple standardization of DNA damage in samples by DNA migration values in stained/scored reference samples does not seem as a suitable way of reducing inter-laboratory variation.

Concluding remarks

The original purpose of the hCOMET ring trial was to test $KBrO_3$ as a positive assay control for the comet assay. This part of the trial has been highly successful as the results demonstrate reproducibility and stability of cryopreserved samples in most laboratories. The inter-laboratory variation in DNA migration levels was not an immediate concern because attempts were made to standardize important steps in the comet assay procedure. It should be noted that we do not know if differences in $KBrO_3$-induced Fpg-sensitive sites between laboratories are due to real difference in number of lesions or different DNA migration originating from differences in assay procedures. Unfortunately, this problem is difficult to solve because the comet assay has high sensitivity (i.e. it detects low levels of DNA lesions). For instance, measured levels of oxidatively damaged DNA by chromatographic techniques are higher than by the enzyme-modified comet assay [47]. Measurement of oxidatively damaged DNA by chromatographic techniques in $KBrO_3$ exposed cells is desirable,

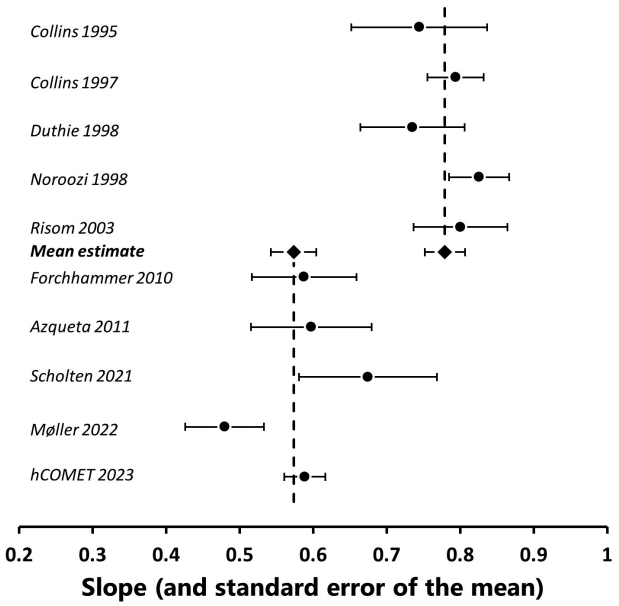

Figure 8. Analysis of relationship between visual score and image analysis (%Tail DNA). The studies are segregated into 'early' [39–43] and "late" studies [16,18,34,44,45]. Slopes are based on linear mixed model with study as fixed (absorbed) factor, visual score as independent factor and %Tail DNA as dependent factor.

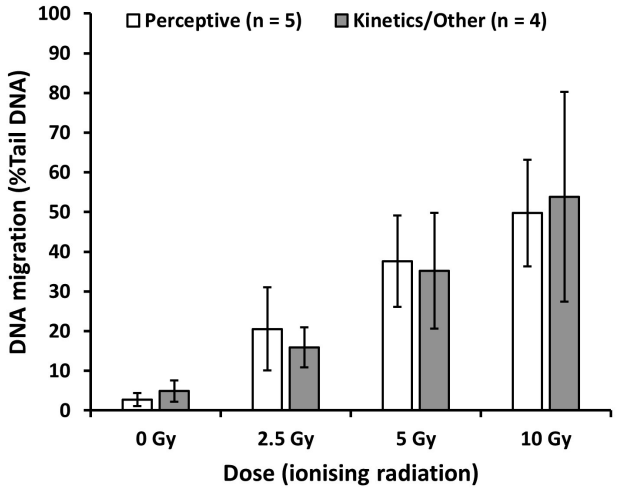

Figure 9. Comparison of DNA migration values of comets in premade slides in the ECVAG trial. THP-1 cells were irradiated with ionizing radiation (0, 2.5, 5, or 10 Gy) and processed in a central laboratory. Slides from the same comet assay experiment were subsequently shipped to participating laboratories. Each investigator/laboratory scored 50 comets from two different gels. The results are mean (columns) and standard deviations (error bars) of DNA migration measured by software from Perspective (five laboratories) or other software (four laboratories using Kinetics and two laboratories using other image analysis systems). The graph has been made from previously published results [18].

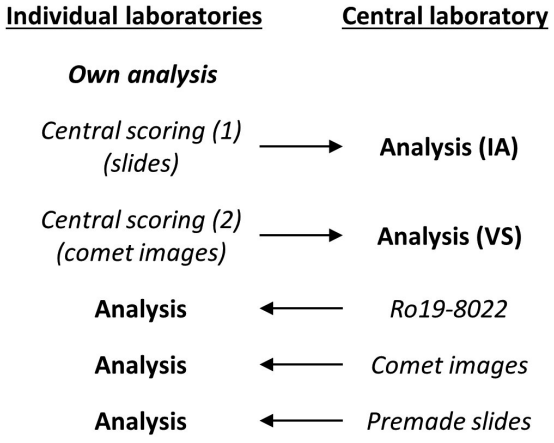

Figure 10. Generation, distribution, and analysis of samples in the hCOMET ring trial. Samples were generated (italic text) and analysed (bold text) in different laboratories. Arrows indicate shipment of samples from individual laboratories to a central laboratory or vice versa. Two different central laboratories scored slides by image analysis (IA) or visual scoring (VS). 'Own analysis' are samples of $KBrO_3$-exposed THP-1 cells that have been generated and analysed by the comet assay in the same laboratory.

although the concentrations might need to be higher than the 4.5 mM that have been used in the hCOMET ring trial.

The inter-laboratory variation in reported DNA migration levels by the comet assay remains a concern. Despite the adoption of the comet assay as an Organisation for Economic Cooperation and Development test guideline and multi-laboratory projects like hCOMET, there is still not even consensus on using the same primary comet descriptor. Many researchers favour %Tail DNA among the image analysis descriptors, and visual scoring is regarded as an equally

Table 3. Contribution of cell culture works, comet assay procedure, and image analysis to the overall inter-laboratory variation

Step	Comet images	Premade slides	Central scoring (1)	Central scoring(2)	Ro19-8022	Own analysis	Partial SD[a]
Cell culture procedure	NA	NA	+	+	NA	+	10.9[b]
Comet procedure (minus staining and image analysis)	NA	NA	+	+	+	+	12.3[c]
Staining	NA	+	NA	+	+	+	7.9[d]
Image analysis	+	+	NA	NA	+	+	0.5
Standard deviation[a]	0.5	7.9	11.3	12.1	14.6	18.2	18.2[e]

Contributions to the standard deviation (SD) are indicated with plus (+) or not assessed (NA) by the analysis of the sample set.
aThe partial SD is based on 25% Tail DNA (rf. linear regression analysis in Fig. 11).
bCalculated as $SQRT[(18.2)^2 - (14.6)^2]$
cCalculated as $SQRT[(14.6)^2 - (7.9)^2]$.
dCalculated as $SQRT[(7.9)^2 - (0.5)^2]$.
eCalculated as $SQRT[(10.9^2) + (12.6)^2 + (7.9)^2 + (0.5)^2]$.

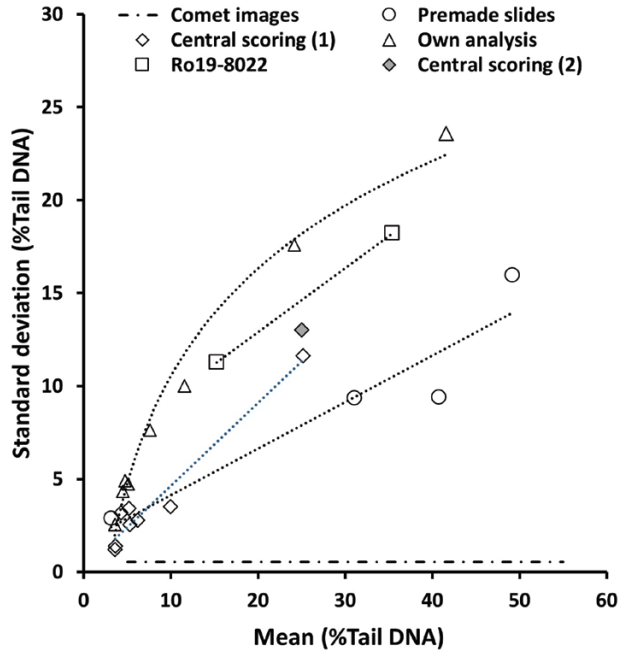

Figure 11. Comparison of inter-laboratory variation in analysis of DNA migration in the comet assay. The graph depicts the relationship between the mean and standard deviation of %Tail DNA values in comet images (i.e. heterogeneity in image analysis), premade slides (i.e. heterogeneity in staining and image analysis; y = 0.25x + 1.6, R^2 = 0.88), central laboratory scoring (1) (i.e. heterogeneity in cell culture procedures and other comet assay steps than staining and image analysis; y = 0.45x + 0.2, R^2 = 0.95), central laboratory scoring (2) (i.e. heterogeneity in cell culture works and other comet assay steps than image analysis; single point estimate), and own scoring (i.e. heterogeneity in the whole comet assay protocol; y = 8.35*Ln(x) −8.7, R^2 = 0.99). Symbols are based on results from 5 to 12 laboratories.

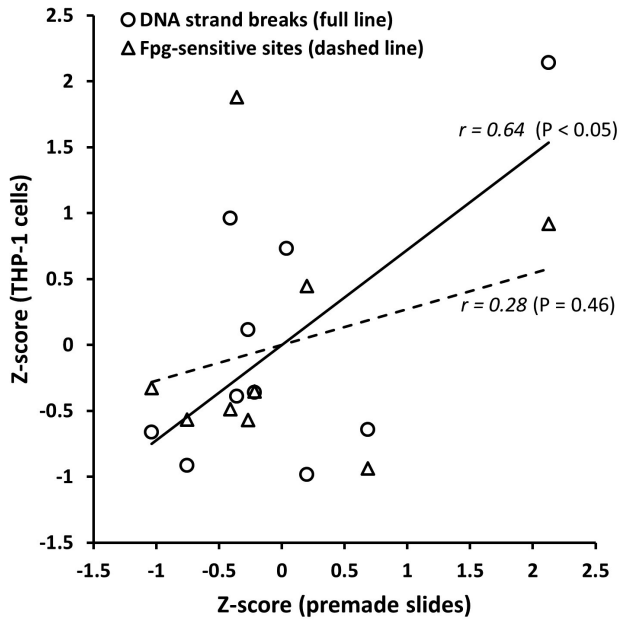

Figure 12. Association between variation in premade slides and $KBrO_3$ exposed THP-1 cells (DNA strand breaks and Fpg-sensitive sites) from the same laboratory. The DNA migration values are normalized to Z-scores within each exposure group. Correlations between levels of DNA migration in premade slides (independent factor) and DNA strand breaks or Fpg-sensitive sites (dependent factors) were tested by linear regression analysis.

good descriptor of DNA migration as %Tail DNA [48]. In fact, visual score and %Tail DNA values are highly correlated [34]. Using %Tail DNA as comet descriptor, a pooled analysis of biomonitoring studies in the hCOMET project showed positive association between the level of DNA strand breaks in leukocytes and risk of premature mortality [49]. However, it was necessary to use a nested design where samples were stratified in tertiles of DNA migration values in each biomonitoring study because of the inter-laboratory variation and use of different primary comet descriptors [49]. Better understanding of crucial steps in the assay procedure and adherence to standard procedures are strong instruments in trials to reduce the inter-laboratory variation. The hCOMET protocols are useful because researchers in different research fields have contributed to their development and free-access tutorial videos accompany the detailed protocols [26,27].

Supplementary data

Supplementary data is available at *Mutagenesis* Online.

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