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## COMMENTARY

# Assessment and reduction of comet assay variation in relation to DNA damage: studies from the European Comet Assay Validation Group

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**The alkaline single cell gel electrophoresis (comet) assay has become a widely used method for the detection of DNA damage and repair in cells and tissues. Still, it has been difficult to compare results from different investigators because of differences in assay conditions and because the data are reported in different units. The European Comet Assay Validation Group (ECVAG) was established for the purpose of validation of the comet assay with respect to measures of DNA damage formation and its repair. The results from this inter-laboratory validation trail showed a large variation in measured level of DNA damage and formamidopyrimidine DNA glycosylase-sensitive sites but the laboratories could detect concentration-dependent relationships in coded samples. Standardization of the results with reference standards decreased the inter-laboratory variation. The ECVAG trail indicates substantial reliability for the measurement of DNA damage by the comet assay but there is still a need for further validation to reduce both assay and inter-laboratory variation.**

In a remarkably short period of time, the alkaline single-cell gel electrophoresis (comet) assay has become a widely used method for the detection of DNA damage and repair in cells and tissue from humans, animals, invertebrates and plants. Using this assay, it is relatively easy to obtain measures of DNA damage induced by well-known genotoxic agents such as hydrogen peroxide and ionizing radiation in almost any cell system. In general, there is considerable knowledge about technical issues such as how the comets are formed and the advantages of various applications of the assay (1). There is even a considerable body of literature describing statistical issues addressed specifically to design experiments in the comet assay (2). However, researchers will probably have noticed that it is often difficult to compare their own results with those reported in other publications. For instance, there are large differences in the reported values of DNA damage in leukocytes from healthy humans and it is not clear whether this is due to different comet assay protocols or real biological differences in different countries (3). The most important reasons for this problem are the lack of standard assay conditions and the results being reported in units that cannot be directly compared. Many steps of the assay protocol (including slide preparation and electrophoresis) affect both intra-assay variability and inter-assay reproducibility. Therefore, as yet, there is no consensus about the normal level of DNA damage in cells and tissues, and the measurement of DNA repair activity by comet assay is still in its infancy (4,5). These applications of the comet assay cannot be considered to have been validated thoroughly, although we are clearly moving towards a better understanding of the variation in the comet assay and can begin to take measures to reduce the variation.

Studies on assessments of DNA damage measured by the comet assay have indicated a substantial variation as shown, for instance, by a large study in Cuba where 19 investigators from seven different laboratories analyzed the same slides (6). Assessments of the variation within one laboratory have also shown considerable inter-investigator variation in the scoring of slides, although the same investigator scored remarkably consistently over time (7,8). Still, the most authoritative study to date has been the European Standards Committee on Oxidative DNA Damage (ESCODD) that had the validation of measurements of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) as the primary aim, for which the comet assay was one among many techniques used. Enzymatic digestion of DNA by formamidopyrimidine DNA glycosylase (FPG) in the comet assay is considered appropriate for the measurement of oxidatively damaged DNA in terms of 8-oxodG and ring-opened nucleobases, such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde). The participants in ESCODD were instructed to follow an approved protocol for the detection of DNA damage. On two different occasions, ESCODD partners received cryopreserved coded samples of HeLa cells for the detection of background levels of FPG sites by the comet assay (Figure 1). The data indicated that there were substantial differences in the levels of DNA damage detected by different laboratories, whereas there appeared to be some concordance in the intra-laboratory values. A more discouraging finding was the observation that only about a half of the laboratories could detect a concentration-dependent increase in FPG-sensitive sites in coded samples (9). The ESCODD project also attempted to determine background levels of FPG sites in human lymphocytes (10). For this purpose, each partner collected blood from healthy humans from their own country and analyzed the cells for FPG sites; these were analyzed together with standard samples in order to adjust for differences between laboratories. As shown in Figure 2, there was a strong association between the level of DNA damage in the blood and reference standard samples. This indicates that the differences in assay procedures, despite the attempt to used standardized protocols, were factors strongly influencing the determination of the levels of DNA damage and thus the variation in DNA damage reported by different laboratories. In a follow up study

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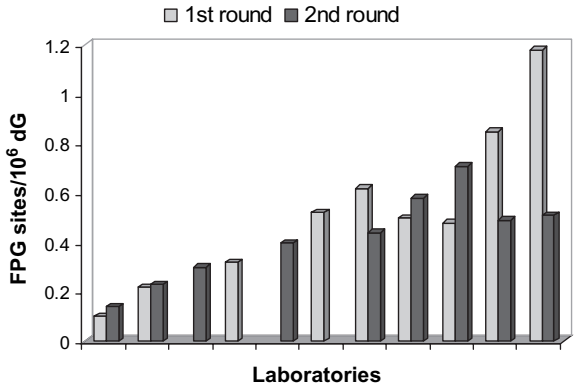


**Fig. 1.** Level of FPG sites in HeLa cells analyzed by different laboratories in ESCODD. The cells were distributed on two different occasions and represent the background level of FPG sites. The coefficient of variation was 57% (9) and 66% (10) in the rounds of analysis. The figure has been composed from original data from each round of analysis, obtained from graphs in the two different ESCODD publications (9,10).

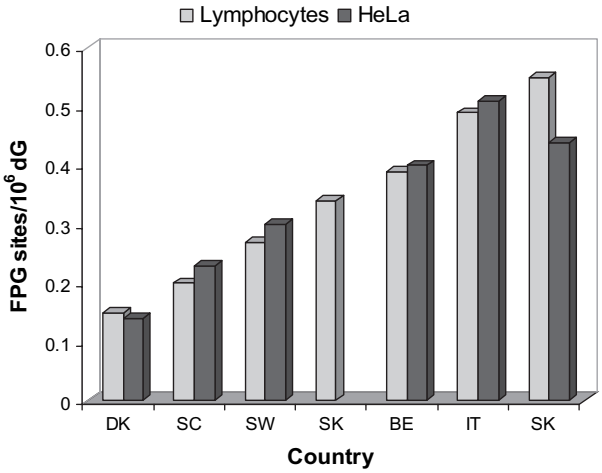


**Fig. 2.** Mean level of FPG sites in lymphocytes collected in Denmark (DK), Scotland (SC), Sweden (SW), Slovakia (SK), Belgium (BE) and Italy (IT). Identical batches of HeLa cells were analyzed in the different laboratories. The figure has been composed from original data obtained from two different graphs in the original ESCODD publication (10).

of young healthy males and females, an attempt was made to establish a normal background level. One interesting result was that females had a significant lower level of lesions when compared to males (11).

The European Comet Assay Validation Group (ECVAG) was established to validate the comet assay with respect to measures of DNA damage formation and its repair, with special focus on its application in biomonitoring studies. The approach was based on the notion that it is difficult to fully standardize the comet assay conditions and that researchers are reluctant to change routine procedures within their laboratories. However, it should be possible to reduce the inter-laboratory variation by using reference samples in the assay. Any DNA-damaging agent could be used as reference standard samples; the ECVAG project used ionizing radiation, which has the advantage that the primary comet assay end points can be converted to the number of lesions per unaltered base pairs.

Two reports in *Mutagenesis* accompanying this Commentary describe the ECVAG assessment by 12 laboratories of variation in level of (i) DNA damage measured by the simple version of the comet assay (lesions commonly referred to as strand breaks) (12) and (ii) oxidatively damaged DNA (detected as FPG-sensitive sites) (13). A third report, on the measurement of DNA repair incision activity, is in preparation. The results from the measurement of DNA damage showed that all laboratories detected a concentration-dependent relationship in coded samples that had been treated with increasing doses of ionizing radiation (12). This finding was reassuring, but there were still substantial differences in the reported damage. The inter-laboratory variation was reduced by adjusting the values with the calibration curve samples; but more importantly, this assessment pinpointed that image analysis rather than the laboratory procedure was an important source of the inter-laboratory variation (12). The assessment of the FPG sites not only showed that all laboratories could detect a concentration-dependent relationship in coded samples but also indicated a large inter-laboratory variation, which was decreased by adjustment of the values using calibration curve samples (13). In general, the ECVAG trial indicates that the quality of measurement of FPG sites by the comet assay has increased substantially since the last assessment in the ESCODD trial, but inter-laboratory and inter-assay variation is still a concern as it affects the statistical power of the assay.

It is well known among comet assay users that there can be a large variation in DNA migration between gels in the same experiment or even between different areas of the same gel. This heterogeneity will give rise to variation if the reference standards are analysed in different gels to the samples, i.e. as external standards. ECVAG has made progress towards developing true internal standards for the comet assay (14), consisting of cells that have had their DNA thymidine substituted with bromodeoxyuridine (BrDU). Using a fluorescent anti-BrdU antibody, plus an additional barrier filter, comets derived from these cells could be readily distinguished from the 'test' cell comets, present in the same gel. The unambiguous distinction between test and reference cell comets enables the reference cell comets to be selectively analysed in an extra round of image analysis. Using the reference cell comets as internal standards led to substantial reductions of the variation for intra- and inter-experimental measures of comet formation and DNA damage repair; only minor reductions in the variation were noted when the reference and test cell comets were in separate gels. These studies indicate that differences between individual gels contribute appreciably to assay variation and that using the reference cells as internal standards allowed greater significance to be obtained between groups of replicate samples.

Continuing ECVAG studies aim to measure the magnitude of the inter-individual variation of oxidatively damaged DNA in white blood cells. There are data suggesting that the inter-individual variation in DNA damage is substantially smaller than the assay variation (15). With this in mind, a reliable assessment of the magnitude of inter-individual variation as well as a measure of the variation in background level of oxidatively damaged DNA in white blood cells between different geographical regions necessitates an assessment of the intra- and inter-laboratory variation. Instead of introducing a standard operating procedure, the ECVAG will focus on developing a set of 'reference conditions' for the comet assay, where a few of the most important steps are kept constant.

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