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Review

The comet assay as a tool for human biomonitoring studies: The ComNet Project

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ABSTRACT

The comet assay is widely used in human biomonitoring to measure DNA damage as a marker of exposure to genotoxic agents or to investigate genoprotective effects. Studies often involve small numbers of subjects, and design may be sub-optimal in other respects. In addition, comet assay protocols in use in different laboratories vary significantly. In spite of these difficulties, it is appropriate to carry out a pooled analysis of all available comet assay biomonitoring data, in order to establish baseline parameters of DNA damage, and to investigate associations between comet assay measurements and factors such as sex, age, smoking status, nutrition, lifestyle, etc. With this as its major objective, the ComNet project has recruited almost 100 research groups willing to share datasets. Here we provide a background to this project, discussing the history of the comet assay and practical issues that can critically affect its performance. We survey its diverse applications in biomonitoring studies, including environmental and occupational exposure to genotoxic agents, genoprotection by dietary and other factors, DNA damage associated with various diseases, and intrinsic factors that affect DNA damage levels in humans. We examine in depth the quality of data from a random selection of studies, from an epidemiological and statistical point of view.

Contents

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1. Introduction

The epidemiological study of cancer and most chronic diseases depends increasingly on the use of molecular biomarkers, i.e. cellular or biochemical parameters that can be measured in a relatively non-invasive way, and that typically give an indication of an individual's occupational or environmental exposure to mutagenic or carcinogenic agents. Biomarkers can also provide information about individual differences in resistance to such agents, whether the result of genetic factors, or so-called adaptive responses, or – for example – behavioural factors including diet and exercise. Biomarkers can in some cases detect early stages in the development of disease, or indicate risk of future disease.

Measuring DNA damage in lymphocytes may be a way to assess the risk of cancer and other chronic diseases for an individual, although many other factors including epigenetic changes modulate the DNA damage response. As a consequence of this uncertainty, several questions arise when setting research priorities:

1. What kind of damage should we look at? Oxidative damage? Strand breaks? Alkylation? Cross-links? Bulky adducts? Chromosome aberrations? Which of these are most important in terms of disease risk?
2. How should we measure the damage? Analytical chemical methods (GC-MS, HPLC)? ^{32}P-postlabelling? ELISA? The comet assay? Certain assays are more appropriate for different lesions. With regard to measuring oxidised bases in DNA, for instance, while chromatographic methods are very precise when measuring high levels of induced damage, when applied to low-level background DNA oxidation, they suffer from the serious artefact of further oxidation occurring during sample preparation. In contrast, the comet assay (single cell gel electrophoresis – in conjunction with the enzyme formamidopyrimidine DNA glycosylase (FPG) to detect oxidised purines, as described below) uses a gentle procedure with little risk of spurious oxidation, and so turns out to be more accurate than the chromatographic assays [1–3].
3. How constant is the level of DNA damage in an individual? Would we really expect a DNA damage estimate at a particular time-point to reflect or predict the long-term risk of disease? Increased chromosome damage – whether aberrations seen in metaphase, or micronuclei (MN) detected in interphase cells – has been associated with genomic instability and cancer risk [4,5], but the links between DNA damage, chromosome damage, and cancer have to be further investigated.
4. It is customary to regard DNA damage measured in human cells as a marker of exposure rather than of long term health effect. Is this the limit of its usefulness?

This paper is concerned specifically with the comet assay – a relatively simple method for measuring DNA damage at the level of individual cells. Originally devised in the 1980s to measure DNA strand breaks (SB) [6,7], it was soon modified to detect a variety of other lesions, such as oxidised bases [8,9]. Because of its simplicity, sensitivity and speed, it has been widely adopted as a biomarker assay in human studies, for example monitoring occupational exposure to mutagens, testing dietary supplementation with antioxidants, or checking levels of oxidative stress in relation to diverse diseases [10].

The recently launched ComNet project aims to investigate whether the comet assay is a reliable, validated biomarker assay that can be used in human biomonitoring [11]. Data will be collected from existing studies for pooled analyses, and protocol features that may affect results will be examined. The construction of a set of widely acceptable guidelines may help to eliminate much of the experimental variation that has generated the large heterogeneity of comet assay data, and that frustrates attempts to compare or combine studies carried out in different laboratories.

2. The comet assay: nearly 30 years of evolution

What we now know as the comet assay has its roots in work done in the 1970s by Peter Cook and colleagues. They described 'structures resembling nuclei but depleted of protein. . .released by gently lysing cells in solutions containing non-ionic detergents and high concentrations of salt' [12]. These nucleoids, they found, became surrounded by a halo of DNA when either irradiated with X-rays or incubated with the intercalating dye ethidium bromide. A model was developed in which the DNA is constrained as, in effect, supercoiled loops attached to a nuclear skeleton or matrix or cage, with functions such as replication proposed to occur at the sites of attachment [13]. Relaxation of supercoiling by a SB allows a DNA loop to expand into the halo.

Ostling and Johanson [6] were aware of this work when they published their report of the electrophoresis of lysed cells embedded in agarose – the first comet assay paper (though they did not use the term, which was introduced by Olive et al. a few years later [14]). The pH of lysis and electrophoresis buffer was 9.5, and relative tail fluorescence was measured with acridine orange as stain. The effect of a γ ray dose of about 0.2 Gy was detectable and saturation of effect was reached at about 3 Gy. They describe the relaxation of supercoils resulting in '*more pronounced migration of DNA towards the anode*'. Singh et al. [7] independently developed a similar method, but with an alkaline unwinding step, and electrophoresis at high pH. They detected the effect of an X-ray dose of 0.25 Gy, with saturation becoming apparent around 2 Gy.

The comet assay in the alkaline form was quickly adopted for use in genotoxicity testing, both in vivo and in vitro. For in vivo work, it has the advantage that it can be applied to virtually any tissue that can be disaggregated into single cells.

An additional step was introduced in the assay, to allow detection of damaged bases as well as SBs. After lysis, the nucleoids are incubated with a lesion-specific endonuclease which converts base lesions to breaks. Endonuclease III (EndoIII) detects oxidised pyrimidines, and it was first applied to human lymphocyte samples in a supplementation trial to demonstrate protection of DNA against oxidation [15]. Subsequently, FPG has been particularly useful in biomonitoring, allowing estimation of the background level of oxidised purines (principally 8-oxoguanine) in human cells [10]. The human homologue of FPG, 8-oxoguanine DNA glycosylase (hOGG1), has also occasionally been employed [16,17].

A few studies have shown that it is possible to detect changes at the level of DNA methylation in single cells, by using methylation-sensitive restriction endonucleases such as HpaII and MspI in combination with the comet assay; either global [18] or gene-specific [19] DNA methylation is detected.

Comets from cells exposed to DNA-damaging agents often show a continuum of DNA migration from cells with little migration (small tails) to cells with almost all DNA in the tail. The latter are often referred to as "clouds" or "hedgehogs". While it was assumed in the past that such comets represent dead (apoptotic/necrotic) cells, it is now clear that they simply represent cells with relatively high levels of damage (but still potentially repairable, and consistent with viability) [20]. Hedgehogs have been observed in experimental studies in vitro and in vivo but it is unclear whether they can be expected in human biomonitoring studies when exposure to DNA-damaging agents is not extremely high. If they occur, they should be scored and reported.

Most human comet assay investigations have used isolated lymphocytes (strictly speaking, peripheral blood mononuclear cells – PBMC), which are relatively easy to obtain. But it is important to have access to other cell and tissue types. Buccal epithelial cells have been used [21], as well as sperm [22], nasal epithelial cells [23], cells from lens epithelium removed with cataracts [24], and biopsies removed during clinical examination or surgery [25].

Crucial to the maintenance of DNA stability, and of relevance to cancer prevention, is the capacity of cells to carry out DNA repair. Both Ostling and Johanson [6] and Singh et al. [7] performed repair experiments, monitoring the decrease in DNA breaks (i.e. relative tail fluorescence) with time of incubation after irradiation of cells. This approach has been applied in various human population studies, for example with ionising radiation as damaging agent to assess SB repair [26] or with EndoIII to measure base excision repair (BER) of oxidised bases [27]. It is, however, rather demanding to carry out the necessary carefully timed incubations of human lymphocytes in a typical population study in which blood samples are collected from many subjects within a short time frame. Interpretation of such experiments is also problematic, as is discussed by Collins and Azqueta [28]. An alternative approach is the in vitro DNA repair assay [29–31]. A subcellular extract is prepared from lymphocytes or, in a recent development, from tissue biopsies [32], and incubated with a substrate consisting of gel-embedded nucleoids from cells previously treated with either Ro 19-8022 + light (to measure OGG1 activity) or UV(C) (to measure nucleotide excision repair (NER) capacity). Breaks accumulate with time as the extract incises DNA at damage sites, and they are measured with a normal comet assay procedure. Individuals have characteristic BER and NER capacities, and there is a spread of activities among individuals [33] – indicating that this can be a useful biomarker assay for use in human studies, aimed at investigating environmental or intrinsic factors that influence DNA repair [30,31,34,35].

However, it should be noted that these assays for DNA repair measure the capacity of the cells to carry out the initial incision step of excision repair and do not indicate the accuracy of completed repair. Because accuracy of DNA repair may be the decisive factor for the characterisation of genomic stability and cancer prediction [36], the comet assay-based methods measuring incision need to be carefully validated before being used for risk assessment.

Considerable effort has gone into increasing the throughput of the assay, by increasing the number of gels on a slide [37] or by placing up to 96 gels on a plastic film [38,39], and so several hundred gels can be run in a typical tank. But this exacerbates the really labour-intensive aspect of the comet assay, which is scoring. Automated scoring systems in theory solve this problem, but in practice they require a great deal of human supervision and the saving in time is limited.

Quality assurance is essential for valid molecular epidemiology studies, and reference standards should be included in experiments to check for intra-laboratory variation, and to facilitate inter-laboratory comparisons. Attempts have been made to develop a true internal standard by embedding 'sample' and 'standard' cells in the same gel; the standard cells are previously labelled with bromodeoxyuridine, so that they can subsequently be distinguished by differential staining [40].

3. Applications of the comet assay in human studies

3.1. Biomonitoring of environmental and occupational exposure

The comet assay is a valuable tool for quantifying DNA damage in populations exposed to various types and doses of genotoxic agents, and can usefully contribute to the '*biological effect dosing*' of occupational and environmental exposures. Further, when no reliable internal exposure markers are available, the comet assay can help with an early identification of health risks. In the last 20 years the assay has been applied to evaluate DNA damage in human populations exposed to different kinds of xenobiotics (Table 1). Valverde and Rojas [41] reviewed 122 human biomonitoring studies published prior to June 2007. An updated search, in May 2013, revealed 37 manuscripts referring to environmental exposure and 139 to occupational exposure.

Increased DNA damage was found in human populations exposed to air pollution, radiation, and pesticides. Studies of air pollution focused on polycyclic aromatic hydrocarbons (PAH), ozone, benzene, heavy metals, (ultra)fine particulate matter (PM), and passive smoking, including the effects of seasonal variations in sunlight, temperature and ozone [41–47]. Some studies used the comet assay to evaluate the impact of living in the near of a waste incinerator, waste disposal or oil refinery [41,46,48–50]. Studies on environmental radiation included subjects monitored after the Chernobyl accident, or exposed to indoor radon, to high natural radiation levels in certain areas of India [41,51–53], or living in an uranium mineralisation area [54]. The reported relative DNA damage increase in exposed vs. control groups ranged between 1.1- and 7.5-fold [41].

The majority of the environmental exposure papers in which the comet assay is used deal with air pollution and inhalation exposure. The assay proved to be useful, along with markers of oxidative stress, to study the oxidative potential of particulate matter (PM) exposure (reviewed in [42]).

Occupational exposure studies using the comet assay are more common than environmental studies and they cover a broader panel of exposures. No effect was reported from studies on workers exposed to traffic fumes, ozone, butadiene, waste disposal, oxidising hair dyes, PAH, radio frequency radiation, toluene and organic solvents, radiation, cobalt dust, mercury vapours and some pesticides [41,42,55,56]. However, far more studies report increases in DNA damage, in blood cells from workers exposed

Table 1
Application of the comet assay in human studies on environmental and occupational exposure, nutrition and diseases.

Exposure/life style	Comet parameter affected?	DNA breaks	Comet endpoint	
			Oxidative damage	DNA repair/or sensitivity to DNA damaging agent
Environmental exposure in general population				
Air pollution				
VOC, PAH, ozone, PM, UFP, heavy metals, biomass burning, traffic gases (incl diesel), urban area,	Yes	8 studies in [41,43–46,48–50][a]	2 studies in [42]	
near waste disposal/incinerator, oil refinery	No	3 studies in [41]	4 studies in [42,47]	
Smoking: active, passive	Yes	Review [75]	1 study in [41][a]	
Radiation				
Radon indoor, natural radiation, uranium	Yes	1 study in [41,51–53]		
	No	[54]		
Accidental radiation (incl. food)	Yes			$N=1$ in [41][a]
	No	1 study in [41][a]		
Season, sunlight	Yes	3 studies in [41]		
Pesticides				
DDT and metabolites, deltamethrin	Yes	2 studies in [41][a]		
Deltamethrin (antimalaria insecticide)	No	1 study in [41][a]		
Occupational exposure				
Air pollution				
Traffic/urban exposures: PAH, traffic gases/fumes, bus/taxi drivers, police, garagemen, paving	Yes	2 studies in [41,57,58]	1 study in [41], 2 studies in [42]	1 study in [41]
workers, UFP	No	2 studies in [41]	[57]	
Diesel exhaust in mine, coke oven plant, incinerator,	Yes	13 studies in [41,59]	1 study in [42]	
petroleum, oil refinery, Al industry, airport/flight personnel (PAH), rubber	No	5 studies in [41]		
VOCs: benzene, toluene, xylene, styrene, vinlyl chloride,	Yes	4 studies in[41,60]		
formaldehyde, bromopropane, butadiene, carbon disulfide, vinyl chloride	No	14 studies in [41]		
Metals: lead, cadmium, Hg vapours mining site,	Yes	11 studies in [41,62–65]		1 study in [41]
welding, glass workers, plating, smelting (Al, Cd, Co, Cr, Ni, Pb, As), metal dust (Co, Hg); Cr(VI) leather tanning industry	No	2 studies in [41,55,56]		
Asbestos, mineral fibres, silica (pottery)	Yes	1 study in [30,31,35,41]		
Waste, sewage, recycling, diisocyanates,	No	2 studies in [41]		
Pesticides				
Pesticide production, spraying	Yes	14 studies in [41,66–69]		
	No	2 studies in [41]		
Radiation				
Accidental radiation (Chernobyl)	Yes	2 studies in [41]		
Radiation hospital (ionising, ultrasound), radio frequency	yes	8 studies in [41]		
	No	3 studies in [41]		
Radiation in clinical settings	Yes	[70]		
Others				
Pharmaceutical plant workers, anaesthetic/antineoplastic drugs, cigarette factory, car/bus manufacturing	Yes	9 studies in [41,61]		
Oxidation hair dyes	No	1 study in [41]		
Nutrition				
Kiwi, broccoli, gallic acid, soy milk, spinach oronions/cherry tomatoes, multivitamin, vitC + E, vegetarism	Yes		Less [15,35,82–86, 94–96,99]	
Carotenoids, blackcurrant juice, cranberry juice, de-alcoholised wine, prebiotic bread + grean tea, spices, tomato, fruit/vegetables increased use, rutin or kiwi fruit juice	No		No effect [87–93,97,98]	
Disease				
Cancer review		[104]	[104]	[104]
Breast	Yes	[105]	FPG and EndoIII [106]	
	No	[106]		
Cervix	Yes	[107]	[107]	Slower repair [107]
Hodgkin	Yes	[108,109]		Slow or no repair [108]
Lung	No	[111]		
Oesophagus	Yes	[110]		
Prostate	No	[112]		[112]
Alzheimer's disease	Yes	[115,116]	FPG and EndoIII [115,116]	
Parkinson's disease	Yes	[117]	FPG [117]	
Down syndrome	Yes	[119]	[119,120]	
Rheumatoid arthritis	Yes	[118,121,122][a]	FPG [118]	Decreased H_2O_2 repair [122][a], delayed repair [124]
	No	[123]	[123]	[123]
Systemic lupus erythematosus	Yes	[123]		No repair [123], delayed repair [124]
	No		[123]	
Diabetes				
Type II	Yes	[118,125,126, 128,130–135]	EndoIII [118,125], FPG [127,129], FPG and EndoIII [126,128,131]	Low repair [126], decreased repair [128]
Type I and II	Yes	[133]		
Type I	Yes			Increased repair [136][a]
	No	[136][a]		
Hyperlipidemia	Yes	[118]		
Coronary artery disease patients/risk,	Yes	[137–139,141]	FPG and EndoIII	
Cardiac syndrome X	No	[140]	[140,141]	
Radiation sensitive syndrome	Yes	[104]	[104]	[104]

[a] Measured in children.

to volatile organic compounds (benzene, toluene, xylene, styrene, vinyl chloride, PAH, etc.), traffic fumes, diesel exhaust, silica, and adhesives [41,42,57–61]. Increased NA damage was also found in workers exposed to asbestos and mineral fibres [31,34,35,41], metals (welding fumes, arsenic, boron, chromium, nickel, aluminium, cobalt, lead, cadmium, mercury) [41,62–65] or handling pesticides during production, distribution and application/spraying [10,41,66–68].

Organophosphates and pyrethroids, the most commonly used pesticides, are designed to block neurotransmission rather than to be genotoxic. However, studies in which the comet assay was used alongside other cytological methods revealed their genotoxic potential [41,69]. Among other occupationally exposed groups an increase in DNA damage was observed in medical personnel exposed to anaesthetic gases, antineoplastic drugs, chronic low dose radiation, ultrasound and diagnostic X-rays [41,70]. In these studies, the comet assay results closely correlated with the findings of chromosomal aberration, sister chromatid exchange and/or MN assays, which are far more frequently used in the field of occupational exposure [71].

In the studies mentioned above, most often peripheral blood was sampled, and tested either as whole blood or after isolation of PBMC. Non-invasive collection of nasal, buccal and tear duct cells has also been employed, but to a limited extent (e.g. [72,73]). Most of the environmental or occupational human biomonitoring studies recruited fewer than 50 exposed vs. 50 control individuals, resulting in a generally low statistical power, as discussed in a subsequent section. Biomonitoring studies of populations exposed at work generally included non-smokers only, although smoking did not appear to be an important confounding factor for the comet assay (e.g. [74]). A general tendency to show higher levels of DNA damage among smokers is reported in a meta-analysis by Hoffmann et al. [75], although there was large heterogeneity between studies, and the strength and the mechanism of this association has to be investigated in more detail. It is of interest that a similar trend was described in smokers monitored with the MN assay, with a significant association of cigarette smoking with DNA damage present only in heavy smokers [76]. Most environmental studies were carried out on adults, although several studies have demonstrated that the comet assay may be used to assess background environmental or hot-spot exposure in children and adolescents [41,49,50,77–79].

The assay gives an estimate of a very early and rather recent response to genotoxic and oxidative stress agents on DNA integrity. The use of DNA damage frequency to predict the long-term risk for cancer and other health outcomes in healthy subjects requires additional evidence that may be provided by large scale epidemiological studies [80].

3.2. Nutritional intervention studies: effects of phytochemicals

Intensive research over a period of almost 20 years has generated a substantial number of publications on the association between intake of phytochemicals and oxidatively damaged DNA bases in leukocytes (or PBMC) (Table 1). However, several studies are of limited quality because they have not been placebo-controlled, as reported by Møller and Loft [81]. Some of the properly controlled studies have indicated a decreased basal level of EndoIII- and/or FPG-sensitive sites in leukocytes by typically a few weeks of regular ingestion of multivitamin tablets [15], kiwifruits [82], broccoli [83,84], gallic acid [85] and soy milk [86]. However, other studies have failed to find statistically significant changes in EndoIII-/FPG-sensitive sites in blood cells after supplementation with carotenoids [87], blackcurrant juice [88], cranberry juice [89], de-alcoholised wine [90] or green tea, spices and tomato [91]. In addition, two studies had a more complete change in diet with ingestion of 500 or 600 g/day of vegetables or fruit and documented no protection against oxidative damage to DNA in leukocytes [92,93].

It has been speculated that the effect size may depend on the nutritional status of the population that is enrolled in these studies [81]. In particular, subjects who have oxidative stress may experience a beneficial effect of antioxidant or phytochemical intervention, although this is not firmly established experimentally. It is also possible that the time-window of effect is important. This was convincingly shown in one placebo-controlled study where ingestion of tablets with vitamin C and E transiently decreased the levels of EndoIII-/FPG-sensitive sites in PBMC of smokers [94]. Other studies have also shown reduced levels of EndoIII-/FPG-sensitive sites in PBMC 4–8 h after ingestion of spinach or onions/cherry tomatoes [95,96], although the studies couldnot be placebo-controlled. Ingestion of rutin or kiwifruit juice did not affect the level of EndoIII-/FPG-sensitive sites [97,98].

In general it is difficult to identify specific phytochemicals with a strong and reproducible beneficial effect on the basal level of oxidised DNA bases in leukocytes. Lower levels of oxidative base damage were found in PBMC from vegetarians compared with non-vegetarians [99]. Conflicting findings of the association between plasma concentrations of carotenoids and levels of oxidised DNA have been reported [87,100,101]. Generally, it is very difficult to conduct nutrition-related molecular epidemiology studies as so many confounding factors may influence the level of DNA damage. However, Staruchova et al. [35] showed, in an unusually large study (383 subjects), that intake of fruits and vegetables inversely correlated with oxidative damage to DNA. Overall so far, the dietary intake of fruits and vegetables seems to be a (weak) predictor of basal levels of EndoIII-/FPG-sensitive sites in leukocytes.

3.3. Ageing, and intrinsic factors

The term ‘intrinsic factors’ refers to individual biological variables which – evidence suggests – might influence susceptibility to disease. Examples are phase I and II metabolism, antioxidant status, and DNA repair capacity. Inter-individual variation in these intrinsic factors may arise from genetic polymorphisms, epigenetic regulation, or environmental influences [10].

DNA repair has been assessed in relatively few studies so far, and there is little evidence for any substantial influence of environmental or occupational exposure, though repair capacity has responded to nutritional supplementation in some but not all trials [28]. A recent study showed that the disease-associated Ogg1 polymorphism was associated with higher levels of FPG- and hOGG1-sensitive sites in PBMC [16].

Dusinska et al. [34], studying 388 subjects (exposed to asbestos and mineral fibres as well as reference groups), found an inverse correlation between activity of the phase II enzyme glutathione S transferase (GST) and oxidised bases measured as EndoIII- and FPG-sensitive sites, and also an association with BER capacity. Staruchova et al. [35] in the same cohort found negative correlations between DNA repair and both glutathione peroxidase (GPx) and catalase. The interplay between DNA damage signalling pathways, biotransformation enzymes, and the regulation of DNA repair is a topic attracting increasing interest [34].

It is generally assumed that DNA damage accumulates with age, and that therefore the comet assay should show higher levels of SBs and/or oxidised bases in PBMC from older age-groups. In fact, results are not consistent, with not all studies showing an age-related increase of DNA damage [10,35,102]. Lymphocytes are the daughters of progenitor stem cells which survive throughout a person’s life-span reflecting the accumulation of genetic defects in

the stem cells, and in turn influencing genome maintenance mechanisms. An increase in DNA damage with age might indicate genetic instability, for example caused by a decline in repair capacity. However, the evidence for this parameter also is conflicting, with reports alternatively indicating no effect of age on repair, a decrease or even an increase in older age classes [28]. Caution should be exercised when interpretating results from single studies. The experience from the collaborative HUMN (HUman MicroNucleus) project shows that only the pooled analysis of large multicenter datasets allowed the drafting of a reliable age-related curve of genome damage as measured with the MN assay [103].

3.4. Links with cancer and other diseases

DNA oxidation products such as oxidised purines or pyrimidines have been measured as putative indicators of the link between DNA damage and cancer. The potential use of the comet assay for DNA damage and repair activity associated with cancer was reviewed by McKenna et al. [104]. More recent studies also show associations of high levels of DNA damage with different types of cancer, including breast [105,106], carcinoma of the cervix [107], Hodgkin's disease [108,109], and esophageal cancer [110] (Table 1). However, lung cancer patients [111] and prostate cancer patients [112] did not differ from controls in levels of damage.

It is important to note at the outset that an association between DNA damage and disease established from case–control studies, however well conducted, is only an association. It is impossible to say whether elevated DNA damage is a cause or an effect of the disease. It appears that inflammation and oxidative stress associated with colorectal cancer progression cause increases in DNA damage (8-oxoguanine as well as ethenoadducts), but also affect repair enzyme activities, in both tumour and normal tissues, in complex ways [113,114]. To establish causality, prospective studies need to be conducted, determining whether individuals with a high level of DNA damage go on to show a higher risk of a particular disease. Such investigations have not been carried out for DNA damage measured with the comet assay – in contrast to the studies showing significantly higher mortality and cancer risk in individuals with high levels of chromosome aberrations [4] or MN [5,76].

Oxidative stress seems to play a major role in the pathogenesis of neurodegeneration and higher levels of oxidised purines were found in the PBMC or leukocytes of Alzheimer's [115,116] and Parkinson's disease patients [117]. Oxidised DNA bases can be useful markers in many chronic diseases where oxidative stress is implicated either as cause or effect, such as diabetes, rheumatoid or vascular diseases [118]. Several studies have demonstrated increased levels of DNA oxidation in Down's syndrome patients [119,120], and increased sensitivity of cells to DNA-damaging agents.

Rheumatoid arthritis (RA) has been shown to be associated with increased DNA damage and impaired DNA repair in PBMC [118,121,122]. An increased level of DNA damage was also found with the comet assay in neutrophils from systemic lupus erythematosus patients, together with an impaired ability to repair oxidised DNA lesions [123,124].

The greatest number of comet assay studies of chronic disease (around 30) have been conducted on diabetic patients, most of them showing elevated levels of DNA damage associated with the disease. Higher levels of SBs or oxidised lesions were found in PBMC of type II diabetics and patients with neuropathy [125–132]. FPG-sensitive sites seem to represent changes specifically related to hyperglycemia, and a strong correlation with serum glucose concentrations was confirmed in several studies [125,129,130,132]. Diabetic patients displayed higher susceptibility to H_2O_2 and to doxorubicin, and decreased efficacy in repair of DNA damage induced by these agents compared with healthy controls [128]. Sardaş et al. [133] found that non-insulin-dependent patients had higher levels of DNA damage compared to insulin-dependent patients. Supplementation with vitamins E and C had an impact on the level of DNA damage. Higher DNA damage and malondialdehyde (MDA) levels in diabetic patients compared to healthy subjects were reported in several studies [134,135], and a negative correlation of DNA damage with superoxide dismutase (SOD) levels [135] or with total antioxidant status [134] was found. Treatment of diabetic patients with statin [134] or gliclazide [126] was associated with a decrease in DNA damage compared with untreated patients.

A few studies have investigated patients with type I diabetes, generally showing higher oxidative stress in diabetics [136]. Diabetic children had increased DNA repair capacity in PBMC (compared with healthy children or corresponding adult patients), perhaps in response to the permanently elevated state of oxidative stress [136]. FPG-sensitive sites correlated with blood glucose in diabetes type II subjects [125]. Impaired antioxidant defence in diabetic patients may be one of the mechanisms responsible for increased DNA damage.

Lipid oxidation is instrumental in the formation of arterial plaques in cardiovascular disease, and PBMC can be regarded as surrogate target cells for the assessment of oxidative stress; so DNA damage measurement can have wider implications than simply a concern for genotoxicity [118]. Demirbag et al. [137] found increased DNA damage and decreased antioxidant status in 53 patients with angiographically documented coronary artery disease (CAD) compared with 42 subjects with normal coronary vessels. Gur et al. [138] also found increased PBMC DNA damage in 23 cardiac syndrome X patients. A study of 120 patients with coronary heart disease and an equal number of matched healthy controls showed a strong correlation between comet tail length and MDA and nitrite/nitrate levels [139]. Oxidatively damaged DNA in PBMC of 40 CAD patients was increased compared with controls and correlated with the severity of the disease [140]. Increased oxidation of lipids, proteins and DNA was seen in 30 patients with CAD, and PBMC DNA damage was found to be more reliable than MDA or protein carbonyls as an indicator of the severity of vascular lesions [141].

The use of the comet assay to analyse repair of DNA damage opens a new perspective for the clinic. Several severe ionising radiation sensitivity syndromes are associated with defects in single or most likely double SB repair. Increased exposure to ionising radiation from occupational exposure or diagnostic medical procedures has raised concern that some individuals might be at an elevated risk from the harmful impact of acute or chronic low dose radiation exposure. Thus, the comet assay appears to be well suited to screen for radiosensitive individuals who may be exposed to ionising radiation during routine clinical procedures [104]. A summary of results concerning the use of the comet assay in populations affected by chronic diseases is given in Table 1. The concordant direction of associations for substantially all diseases investigated confirms the great potential of this assay for clinical practice.

4. Practical issues – from blood sampling to comet scoring

4.1. Issues related to sample collection, management, and storage

Several sources of variability are present in human biomonitoring studies, and the availability of details about individual characteristics becomes a critical factor in handling and interpreting data from a human trial.

Seasonal variation in the sampling period has been shown to impact on comet assay results [142], and this has been used to

generate an exposure gradient in some studies on UV radiation [143,144]. However, variation over time can be a problem in biomonitoring studies, as can the time elapsed between sampling in the field and either storing the sample or conducting the comet assay. A long delay may allow DNA damage to be repaired, unless blood is kept cold (at 4 °C rather than on ice). On the other hand, if studying background exposure levels, keeping blood at 4 °C after blood collection may give an increase in damage compared to storing it at room temperature. The choice of anticoagulant may be crucial, as an increase in plasma DNA (from damaged leukocytes) is reported to occur beyond 6 h in samples collected with citrate or heparin [145]. Whether the choice of anticoagulant has any effect on comet assay results is not clear; only anecdotal reports are available.

In most cases, blood is sampled from a vein in the arm, though finger prick samples can also be used if a small yield of comets is acceptable. The first major variation in technique is the use of either whole blood or the fraction of leukocytes isolated by centrifugation over Lymphoprep (or equivalent). Lymphoprep separates mononuclear cells, i.e. monocytes and lymphocytes. Nucleated cells in whole blood mainly consist of neutrophils (60–75%) followed by lymphocytes (20–30%), whereas Lymphoprep preparations consist of 95–98% lymphocytes. Since neutrophils are short-lived cells (up to a few days), the analysis of DNA damage in whole blood may not be comparable with that performed on the relatively long-lived lymphocytes obtained from Lymphoprep preparations, especially when exposure to xenobiotics took place several days before the analysis. An additional confounding factor is the different sensitivity of white blood cell subpopulations. Morillas et al. [146] reported that T lymphocytes were more susceptible to H_2O_2 than the remaining T lymphocyte-depleted whole blood. It was further confirmed by Wojewodzka et al. [147], who showed that T lymphocytes were more sensitive to ionising radiation than were B lymphocytes. On the other hand, it has been reported that mechanical isolation of lymphocytes may result in increased DNA damage that could account for variability in results [123]. All these factors must be recognised as limitations of the assay, in particular when a similar exposure is compared using whole blood vs. isolated PBMC. Inclusion of a whole blood differential count in the study might help to control for bias caused by different white blood cell composition.

Using whole blood is obviously simpler, but leads to a less homogeneous cell population and possible problems due to interference from the presence of red cells, in challenge and repair assays. A common experiment involves treating cells with H_2O_2 in order to assess their resistance to oxidant challenge (reflecting antioxidant status), but when this is applied to whole blood, virtually no DNA damage is induced, perhaps because the peroxide is immediately broken down by catalase from the red cells. For other applications, this might however be an advantage, as possible additional DNA damage – induced during the PBMC isolation procedure – is avoided [148]. Both whole blood and isolated PBMC can be frozen and stored at ~−80 °C or in liquid nitrogen. Whole blood is recommended to be diluted with an equal volume of culture medium containing 20% DMSO [149]; PBMC are suspended in PBS or medium with 10% DMSO. The DMSO prevents damage during freezing, which is normally done slowly – around 1 °C per minute or less. However, a recent paper [150] describes the fast freezing of small aliquots of whole blood with no DMSO – giving impressively low levels of basal DNA breakage in subsequent comet assay analysis. The small volume appears to be critical, though it is not clear why this should prevent cell and DNA damage; perhaps because fast freezing of a small volume avoids crystal formation [151].

Thawing the cells is another critical stage. Fast thawing and immediate dilution with PBS, centrifugation and resuspension are necessary to preserve DNA integrity. Excessive damage, whether from sub-optimal freezing or thawing, renders samples unusable. A certain low level of damage induced by handling may be acceptable, although it will increase the 'noise' in results. A threshold level of 'normal' (or acceptable) DNA damage should be established within each laboratory, possibly based on historical positive and negative control data.

Because PBMC from venous blood are by far the most commonly used type of cell in human biomonitoring studies employing the comet assay (91% of studies reviewed for this paper), issues relating to blood cells have been the main focus here. However, as noted earlier, other cells have been used [21–25]. In particular, buccal cells, which can be collected from the inner cheek of the mouth by mouthwash or by gentle brushing with a soft toothbrush, offer a potentially useful and attractive option [21,152]. These cells are easily and non-invasively collected and reflect DNA damage in epithelial cells, an important type of target cell for biomonitoring. The buccal cell approach has not been widely used to date in comet assay testing, but is worthy of further study, and optimised conditions have been described [153]. The use of the comet assay in buccal cells and in other specimens, such as exfoliated cells from nasal mucosa or tear ducts [154,72] will be specifically addressed in a coming publication of the ComNet collaborative group.

4.2. Effects of variations in the comet assay protocol

Cell density is the first critical feature to take into consideration; clearly too few comets may jeopardise an experiment, but also too many cells can present problems, since overlapping comets are difficult to score accurately. For the conventional comet assay with 1 or 2 gels per slide, 10^4 cells per gel is an appropriate number. The cell count is routinely done before freezing the cells. Ho et al. [155] recently showed that the yield of cells steadily decreases during the stages up to embedding in agarose, and so the actual number of cells per gel is probably a lot less than 10^4.

The next variable to be considered is the agarose concentration. At 0.4% or less, the gel is very fragile, and between 0.4% and 1.3% there is a progressive decrease in tail intensity of comets from γ-irradiated or H_2O_2-treated cells [156,157]. At much above 1%, migration of the DNA is seriously impeded. It is not uncommon for cells to be centrifuged, resuspended in the drop of supernatant remaining, and then mixed with agarose. The volume of this drop is not controlled, and so inconsistent final agarose concentrations can result.

The lysis solution is essentially >2 M NaCl plus a detergent. Triton X-100 is almost always present. Sometimes sodium sarcosinate is added, though it seems unnecessary, since cell and nuclear membranes are broken by Triton X-100 alone. DMSO is quite often added to the lysis solution, although the justification for this is vague (if whole blood is being used, DMSO might be useful, to protect against iron-catalysed oxidative damage). Lysis time is invariably 1 h or more. No deleterious effects of a longer lysis have been reported.

After lysis, an additional step may be a digestion with lesion-specific endonuclease, to detect damage in addition to simple SBs. This is a stage that needs careful control of the enzyme concentration, time and temperature of incubation. Ersson and Möller [156] have recently optimised the digestion conditions for FPG, but this should ideally be done in each laboratory using the enzyme. It is important to include samples incubated with buffer alone (without enzyme) as well as positive control cells, treated with Ro 19-8022 plus light or $KBrO_3$ (to induce base oxidation) and incubated with and without enzyme.

Before electrophoresis, slides are placed in cold alkaline solution for a variable time. Ersson and Möller [156] and Azqueta

et al. [157] independently examined the importance of this step. Both found 40 min to be satisfactory, though longer incubation might be required to convert alkali-labile sites quantitatively to breaks. Although enzymes such as FPG and OGG1 combine a glycosylase (removing the damaged base and creating an apurinic/apyrimidinic or AP site) with an AP lyase (converting the AP site to a break), it is not clear whether the AP lyase activity is sufficient, or whether the subsequent alkaline incubation completes the process of conversion.

The voltage applied during electrophoresis, as well as the time of electrophoresis, are both critical parameters [156,157]. The voltage gradient should be measured on the platform carrying the slides, and not simply between the electrodes, since the voltage gradient is much steeper across the platform than through the reservoirs on each side. About 1 V/cm across the platform for 25 min is generally satisfactory; within reasonable limits, either voltage or time can be changed, keeping the product V/cm × time constant.

5. Critical epidemiological and statistical issues

To provide a tentative identification of critical aspects of the study design and statistical analysis of human biomonitoring studies employing the comet assay, an arbitrary set of 50 studies investigating DNA damage in human populations was randomly selected from papers published in the last decade (a table describing individual studies and the list of references is provided as additional material). This exercise allowed us to survey the most critical features in the design and analysis of population studies; subject selection criteria, control for confounding, choice of the DNA damage parameter, population size, statistical power, model fitting, etc.

The heterogeneity among studies selected for this survey is evident, with a large degree of variability in protocols, but also in design, endpoints and scoring criteria.

5.1. Study population

The large majority of biomonitoring studies in our study sample evaluated the effect of occupational exposures (37 out of 50 studies, 74%). Other study groups included subjects affected by disease (mostly cancer) or exposed to DNA damaging/protecting agents (14% and 12%, respectively). In most publications, limited detail is given about the criteria for selecting the study population, especially as far as controls are concerned [158]. The careful matching of controls is particularly critical, and yet in many of the studies evaluated the control population is defined simply as unexposed or healthy subjects. Inclusion and exclusion criteria are seldom described.

5.2. Host factors and confounding factors

The role of potential and actual confounders must be evaluated both in the design and in the statistical analysis of epidemiological studies. Most commonly evaluated factors were age, sex, smoking and drinking habit, exercise, and drug intake, while inclusion and exclusion criteria were seldom reported. Unexpectedly, 12 studies in our sample (24%) did not take into consideration any possible confounders.

As reported by the WHO International Programme on Chemical Safety (IPCS), confounding can easily be generated during the collection and management of biological specimens for several reasons, including the timing of sample collection, lack of blind coding, seasonal differences, etc. These features are only occasionally discussed in the papers we have evaluated

The use of protective devices in occupational studies is occasionally recorded, and different genotypes identified (14.8% and 22.2%, respectively), although these are generally effect modifiers rather than confounders. These observations are in keeping with Møller et al. [158], who reported that age, air pollution, diet, exercise, sex, infection, residential radon exposure, smoking and sunlight are the main factors that influence the level of DNA damage detected by the comet assay in biomonitoring occupational studies.

5.3. Sample size

The evaluation of optimal sample size is a critical aspect in the design of epidemiological studies, since it determines the minimal size of the study population large enough to reach statistical significance if the observed effect size is as expected. The population size of the studies included in our review ranged from 5 to 205 subjects per group, with an overall mean of 52 and more than half of them (66%) evaluating fewer than 50 subjects per group. The small size of most studies, and the lack of consideration for the statistical power of the study, mean that statistical planning must be among priorities to be addressed by collaborative efforts aimed at improving the quality of comet assay biomonitoring studies.

5.4. Data scoring and DNA damage parameter

Comets have a complex form consisting of a head and a tail, the head being composed of intact DNA and the tail consisting of broken DNA loops. After staining of DNA and visualisation by fluorescence microscopy, a comet can be described by one or more parameters summarising its shape and size. Parameters can be obtained both by visual or automatic scoring based on image analysis, and recent studies have demonstrated the validity of both approaches [159]. *Visual scoring* using an eyepiece micrometre allows measurement of DNA migration expressed as tail/image length (μm). Alternatively, following visual examination of slides, comets can be classified using a category classification scheme (for instance the 5-category classification scheme ranging from 0, corresponding to no damage, to 4, corresponding to almost all the DNA in the tail) based upon comparisons with standard images [2]. *Computerised image analysis systems*, by far the most commonly used (78% in our sample) often provide three measures of DNA migration: tail length (μm), tail intensity (% of fluorescence/DNA migrated in the tail), and tail moment, the latter being a non-standard unit calculated, by analogy with the mechanical term (i.e. moment: the product of a quantity and a distance to some significant points connected with that quantity), as % of DNA migrated in the tail (i.e. the quantity) multiplied by the distance of DNA migration in the tail. Despite the increasing use of the comet assay in biomonitoring studies, no consensus has been reached about the comet parameter which more properly describes the extent of the DNA damage. Tail length is only useful when low levels of damage are present, as it reaches a plateau relatively quickly; it is not recommended for biomonitoring purposes. The percentage of DNA in the tail (%tDNA), namely *tail DNA* or *tail intensity*, has been proposed by several authors to be the most generally useful parameter since it uses a quantitative measure of damage (from 0 to 100%). Furthermore, this parameter is less variable across studies [2,160,161]. Tail moment has the disadvantage (fatal for the purpose of inter-laboratory comparisons) that it does not have standard units, and given a particular tail moment it is impossible to visualise the level of damage being described. For all these reasons %tDNA is increasingly considered the preferred metric of DNA strand breakage in the comet assay [2,162]. Thirty-four per cent ($N = 17$) of studies in our sample measured this parameter.

Although image analysis on comets seems to be preferable for continuity in assessing DNA damage, it is not strictly required.

Quantification of DNA migration by visual scoring strictly correlates with %tDNA assessed by image analysis [163], with each visual score grade equivalent to ~20% band on the % tail DNA scale. A total of 11 studies in our sample (22%) chose this method for measuring DNA damage.

5.5. Number of scored nucleoids

Most biomonitoring studies based on the comet assay scored 50 nucleoids per slide and two slides per subject, reaching a total of 100 scored per individual (42 studies out of 50; 84% in our sample). The optimal number to be scored in order to reduce the uncertainty of estimates and obtain statistically significant results has not been properly defined. Lovell and Omori [164] concluded that, while increasing the number of nucleoids measured per unit produces more precise estimates, sample sizes of 50 per replicate are satisfactory.

5.6. Statistical analysis of data

In the group of studies selected for this exercise, various statistical approaches, including parametric, non-parametric, multivariate and especially univariate testing, were applied to evaluate the effect of a specific condition on the comet assay endpoints.

The most common statistical procedures were the Student's *t*-test, employed in 23 studies (46%), ANOVA analysis in 22 (44%), and Mann–Whitney test in 17 (34%). Regression analysis was carried out in 14 out of 50 studies (28%). Only occasionally were standard approaches for epidemiologic studies, such as controlling for confounding and testing for interaction, specifically applied. In general procedures for checking statistical assumptions were not described, and nor was the reason for choosing a particular statistical analysis. Although univariate tests, both parametric and non-parametric, are appropriate for most study purposes, the full control of confounding factors and identification of interactions requires statistical modelling [164]. Among most common weaknesses in the statistical analysis of these studies is the use of the *p*-value to compare study groups, while the use of quantitative point estimates of effect, e.g. means ratio of exposed vs. controls is poorly considered. The use of the *p*-value to discriminate significant from non-significant results should be discouraged and quantitative measures of effect used instead. Quantitative measures of effect should be accompanied by confidence intervals, which provide information about the variability and the precision of the observed effect size.

6. Knowledge gaps and research priorities

In human biomonitoring studies using the comet assay, a population generally experiences multiple exposure conditions which need to be taken into account. Ideally, exposure should be assessed in conditions where exposures not related to the study hypothesis are standardised, in order to minimise the number of independent variables. A better knowledge of common exposures to DNA damaging agents and the study of their interaction are among the most urgent priorities to be addressed in any validation studies.

The inevitable presence of intra-laboratory variability requires the inclusion of reference standards in each experiment, especially when a long series of human cell samples are being analysed over a period of weeks or months. The standards for positive control should be aliquots frozen from a single batch of cells, treated with an appropriate DNA damaging agent such as H_2O_2 or ionising radiation (for SBs), or photosensitiser plus light (for 8-oxoguanine). Negative controls should also be run. In this way, anomalous results caused by an experimental problem can be identified; long-term drift (i.e. damage values steadily increasing or decreasing with time) can be spotted; and in many cases deviations can be compensated by normalising results against the standard values.

Another open issue which may be addressed by large collaborative studies is the identification of an 'acceptable' level of basal DNA breakage. Generally, for PBMC, a damage level above 10% DNA in the tail might indicate some DNA breakage occurring during storage, or faulty scoring, or a sub-optimal protocol, although historical data from single laboratories may provide more sensitive thresholds. Consistently high values might actually reflect a genuinely high level of damage in the population being studied, although comparison with the reference standards should reveal the true nature of the elevated damage.

As mentioned above, one of the major problems in comparing results from different laboratories remains the use of different parameters – tail length, %tDNA, tail moment and visual score being the most common. A consensus on the use of %tDNA in biomonitoring projects employing the comet assay would greatly contribute to the standardisation of biomonitoring studies with this assay, and is regarded as a priority [165].

There have been several inter-laboratory trials to date, in which identical samples of cells containing specific damage have been provided for analysis. The results have been generally disappointing. The ESCODD project [3] established the comet assay as a relatively accurate method, although inter-laboratory variability, when measuring 8-oxoguanine in the same material, was considerable. The European Comet Assay Validation Group (ECVAG) was established for the purpose of identifying and reducing inter- and intra-laboratory variations in comet assay measurements of DNA damage and DNA repair incision [166]. While analysis of the same cell samples on different days in the same laboratory showed relatively little variation, there were larger differences between laboratories [156,167,168]. ECVAG implemented the adoption of "standard conditions" to reduce inter-laboratory variation. However, half the laboratories participating to this project apparently were unable to adopt these standard conditions, while results from the successful laboratories indicated some improvement in inter-laboratory variation in measurement of FPG-sensitive sites [169]. There is a continuing need for trials to solve this problem, and the identification of standard conditions for the assay protocol, study design and statistical analysis, remains the most urgent priority to be addressed by any validation efforts.

Finally, the experience from other biomarkers of early genomic damage [4,5,170] has shown how the link between DNA damage and the risk of disease in healthy individuals could be efficiently evaluated by prospective cohort studies.

7. Meeting the challenge – the ComNet project

ComNet – a network of researchers using the comet assay in human biomonitoring studies – was launched during the 9th International Comet Assay Workshop meeting in Kusadasi, Turkey, in September 2011 [11]. ComNet will address the problems that are encountered when comparing comet assay results between laboratories, in the process (we hope) validating the comet assay as a biomonitoring tool. Our objectives are:

- To recruit researchers with an interest in the comet assay to the network (May 2013 over 100 laboratories worldwide registered on the website).
- To collect information about human population studies carried out so far by means of a questionnaire collecting information about technical protocols used, and investigating the availability of epidemiological data. Questionnaires have been contributed from 50 laboratories so far. The geographical distribution of laboratories participating to the ComNet consortium is reported in Fig. 1.

Fig. 1. Geographical distribtion of laboratories participating to the ComNet project (small star 1–2 laboratories, larger star 3 or more laboratories).

- To collect available data from these studies in order to carry out a pooled analysis. This will be a core activity of ComNet, aiming to establish baseline DNA damage levels for future reference, and to investigate associations between comet assay measurements and sex, age, smoking status, nutrition, lifestyle, etc.
- To determine the experimental factors that affect performance of the comet assay and therefore also influence its reliability and robustness as a biomonitoring tool.
- To organise inter-laboratory trials with identical samples, in order to reduce variability and validate the comet assay methods for measuring DNA damage and repair.
- To collect and re-analyse cryopreserved samples from published biomonitoring studies to validate the pooled analysis and to test the extent of inter-laboratory variation.
- To produce a set of practical guidelines for planning and executing population studies with the comet assay, for storing, transporting and analysing samples, and for analysing and interpreting data.
- To explore the relevance of DNA damage and repair measurements to human health and disease.

For further information on ComNet, visit www.comnetproject.org.

Conflict of interest statement

No competing interests.

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