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DNA repair as a human biomonitoring tool; comet assay approaches

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Abstract

The comet assay offers the opportunity to measure both DNA damage and repair. Various comet assay based methods are available to measure DNA repair activity, but some requirements should be met for their effective use in human biomonitoring studies. These conditions include i) robustness of the assay, ii) sources of inter- and intra-individual variability must be known, iii) DNA repair kinetics should be assessed to optimize sampling timing; and iv) DNA repair in accessible surrogate tissues should reflect repair activity in target tissues prone to carcinogenic effects. DNA repair phenotyping can be performed on frozen and fresh samples, and is a more direct measurement than genomic or transcriptomic approaches. There are mixed reports concerning the regulation of DNA repair by environmental and dietary factors. In general, exposure to genotoxic agents did not change base excision repair (BER) activity, whereas some studies reported that dietary interventions affected BER activity. On the other hand, *in vitro* and *in vivo* studies indicated that nucleotide excision repair (NER) can be altered by exposure to genotoxic agents, but studies on other life style related factors, such as diet, are rare. Thus, crucial questions concerning the factors regulating DNA repair and inter-individual variation remain unanswered. Intra-individual variation over a period of days to weeks seems limited, which is favourable for DNA repair phenotyping in biomonitoring studies. Despite this reported low intra-individual variation, timing of sampling remains an issue that needs further investigation. A correlation was reported between the repair activity in easily accessible peripheral blood mononuclear cells (PBMCs) and internal organs for both NER and BER. However, no correlation was found between tumour tissue and blood cells. In conclusion, although comet assay based approaches to measure BER/NER phenotypes are feasible and promising; more work is needed to further optimize their application in human biomonitoring and intervention studies.

Keywords: DNA repair; comet assay; human biomonitoring; validation

1. Introduction

1.1. DNA damage and repair

Human DNA is exposed to both exogenous and endogenous agents that can modify its structure. These structural alterations can take different forms: breaks in the sugar-phosphate backbone affecting one or both strands [i.e., single strand breaks (SSBs) or double strand breaks (DSBs)], oxidation or alkylation of bases, large molecules covalently linked to DNA bases (bulky DNA-adducts), proteins linked to DNA bases (protein-DNA cross links), covalent bonds between bases in the same strand (intra-strand cross links) or in different strands (inter-strand cross links), and wrongly paired bases [1]. These DNA lesions can affect transcription but, more importantly, if not repaired or if mis-repaired before the replication process, they can induce mutations. Mutations in key genes (*e.g.* genes that control DNA repair, DNA replication, cell cycle control or chromosome segregation and apoptosis) are involved in the development of cancer and other degenerative diseases [2].

DNA repair systems, involving different groups of proteins, amend the majority of DNA damages before permanent genome changes can occur. Different DNA repair pathways deal with the various kinds of DNA lesions (see table 1). For instance, SSBs are repaired by the insertion of one or a few bases followed by ligation, while DSBs are repaired by more complicated processes, namely homologous recombination and non-homologous end-joining pathways (the latter being error-prone and therefore potentially mutagenic). Small base alterations such as oxidised and alkylated bases are predominantly repaired by the base excision repair system (BER), involving removal of the damaged base by a specific glycosylase, excision of the resulting baseless sugar, insertion of correct nucleotides using the opposite strand as template and ligation. More complex lesions such as bulky adducts, inter- and intra-strand cross links, and protein-DNA cross links are repaired by the nucleotide excision repair system (NER), in which an oligonucleotide containing the damage is excised and replaced with the correct nucleotides. Finally, wrongly paired bases are repaired by the mismatch repair system. For more details on each DNA repair mechanism, we refer to two comprehensive reviews [1, 4].

DNA repair activity is regarded as a valuable human biomarker, reflecting susceptibility to the accumulation of mutations and thus to cancer, the assumption being that a high intrinsic repair activity will reduce the likelihood of damage being present at replication. Repair activity is frequently assessed by measuring the level of transcription of selected genes from different DNA repair pathways or by the detection of gene polymorphisms (the latter often have

105 unknown consequences). However, the activity of an enzyme does not entirely depend on
106 transcription and DNA repair is actually regulated in a post-translational manner, so a
107 phenotypic or functional assay is more direct. Moreover, phenotypic analyses take into
108 account the influence of environmental factors. Different phenotypic approaches based on the
109 comet assay have been used to monitor DNA repair in human samples, but the question
110 remains whether these assays are suitable for application in human biomonitoring studies.

111 The COST Action hCOMET ('The comet assay in human biomonitoring', CA15132,
112 http://www.hcomet.org) with more than 60 researchers from 25 countries is addressing the
113 application of the comet assay (single cell gel electrophoresis) to measure both DNA damage
114 and DNA repair in human samples. This article has been prepared in the framework of this
115 project as a starting point for further validation trials of the comet assay for assessing DNA
116 repair activity. A working group on DNA repair measurements using the comet assay has
117 identified the required conditions for using DNA repair phenotyping in human biomonitoring
118 studies. These conditions include: 1) technical robustness of the assay; analysis of DNA repair
119 activity by the comet assay must have advantages compared to other techniques; 2) sources of
120 inter- and intra-individual variability must be identified; 3) DNA repair kinetics should be
121 assessed to optimize sampling timing; and 4) DNA repair in accessible surrogate tissues should
122 reflect repair activity in target tissues (i.e. tissues prone to carcinogenic effects). Here we
123 describe the current status of these aspects in the scientific literature.

124 In this review, we predominantly included human biomonitoring studies that focussed on
125 assessing DNA repair activity by comet assay approaches (see next section) in easily accessible
126 tissues or cells. The comet assay-based repair assays are continuously and successfully being
127 validated while at the same time being applied in various research studies. Scientific
128 achievements emerge at the same time as initiatives to understand the assays, improving their
129 reliability, and extending the applications to new tissues. The status of the assays is not
130 advanced to a state where standardized protocols have been adopted. Substantial
131 heterogeneity exists between studies, which very much depends on variation in assay
132 conditions [5]. Although, meta-analysis is an integrate part of systematic reviews, the present
133 variability in the comet assay-based DNA repair assays precludes a meaningful meta-analysis.
134 Thus, only qualitative outcomes of the individual studies will be discussed her.

135

136 **1.1.1 The comet assay**

137 Although the alkaline comet assay (single cell gel electophoresis) was primarily developed as a
138 method to measure DNA damage, it has also been used to measure DNA repair. The standard
139 version of the comet assay measures DNA strand breaks (SBs) in individual cells. The protocol
140 is simple [6]: briefly, cells are embedded in agarose, placed on a microscope slide and lysed to
141 remove membranes and soluble components (including histones) leaving nucleoids (i.e.,
142 supercoiled DNA attached at intervals to a nuclear matrix forming loops) [7]. After that,
143 nucleoids are exposed to an alkaline treatment and to alkaline electrophoresis. The presence
144 of breaks in the DNA relaxes the supercoiled loops and enables the DNA to migrate towards
145 the anode. Finally, DNA is stained with a DNA fluorescent dye and visualized by fluorescence
146 microscopy, revealing images similar to the stellar comets. The more breaks that are present,
147 the more DNA is able to migrate to the anode. The percentage of DNA in the comet tail
148 represents the frequency of DNA SBs and is measured by image analysis. It is worth to mention
149 that DNA cross-links have the opposite effect; they inhibit the migration of the DNA loops.
150 About 50-150 cells (comets) are evaluated per sample and the mean or median value is
151 normally calculated as the descriptor of the sample. Visual scoring methods have been used,
152 though it is currently not the method of choice. In this system, comets are visually classified in
153 5 categories according to the intensity of the comet tail and head [8]. Each comet is given a
154 value between 0 and 4; 0 for undamaged comets and 4 for the comets with almost all DNA in
155 their tail. The overall score is calculated by applying the following formula: (percentage of cells
156 in class 0 x 0) + (percentage of cells in class 1 x 1) + (percentage of cells in class 2 x 2) +
157 (percentage of cells in class 3 x 3) + (percentage of cells in class 4 x 4). Consequently, the total
158 score is in the range from 0 to 400 arbitrary units (AU). This system gives reliable results when
159 applied by an experienced operator and is comparable to the scores obtained using image
160 analysis systems [9].

161 The digestion of the nucleoids (i.e., naked DNA remaining after the lysis of the cells) with lesion
162 specific enzymes allows the detection of other lesions such oxidises bases [10].
163 Formamidopyrimidine DNA glycosylase (Fpg) is the most used in order to detect 8-oxo-7,8-
164 dihydroguanine (8-oxoGua) though it also detects other DNA lesions.

165

166 **1.2 Comet based approaches to measure DNA repair**

167 **1.2.1 Cellular repair assays**

168 The most straightforward approach to measure DNA repair activity is to induce DNA damage in
169 cells and subsequently monitor the rate of repair/removal of these lesions over time.

170 Interestingly, the comet assay was developed to measure DNA repair from the very beginning;
171 followed the reduction in the number of radiation-induced breaks with time, which represents
172 the repair of those lesions [11]. Singh et al., used what is now referred to as a 'challenge assay'
173 or 'cellular repair assay' (as it will be called in the rest of this paper), which follows the kinetics
174 of removal of a certain DNA lesion and re-ligation of the remaining SB with time (Figure 1) [11].

175 SB re-ligation following X- or ɣ-irradiation, or H_2O_2 treatment has been extensively studied in
176 human biomonitoring [12], but it is possible to monitor the removal of other DNA lesions such
177 as oxidised and alkylated bases, and UV-induced cyclobutane pyrimidine dimers, using
178 appropriate enzymes to convert the lesions to SBs [6]. The specificity of the cellular repair
179 assay, regarding the DNA repair pathway that is measured, depends on the DNA-damaging
180 agent, the version of the comet assay (i.e., with or without enzymes) and the substrate
181 specificity of the enzyme used.

182 The advantage of this assay is that the entire DNA repair process is assessed, since it depends
183 on the restoration of the normal DNA structure. Moreover, since DNA repair is measured at a
184 cellular level, the presence of cell populations with different DNA repair capacity can in theory
185 be detected. However, from a technical point of view, it is rather complicated to measure
186 repair in this way, because it requires hours of cell culture and sampling at intervals for comet
187 assay analysis, highly limiting the number of samples that can be analysed at the same time.

188 The fact that cells (normally white blood cells) are under *ex vivo* conditions might also
189 influence the DNA repair process. Although there is no direct evidence to support this notion,
190 the higher *ex vivo* oxygen tension, compared to the *in vivo* conditions, could for instance alter
191 the repair process. Moreover, interpretation of the results is complicated by the fact that DNA-
192 damaging agents may induce different amounts of lesions in different subjects, so that repair
193 starts at different substrate concentrations [12]. This may be particularly important if the initial
194 amount of damage is too high, reaching the point of saturation of the comet assay.

195

196 **1.2.2 Inhibitor-based cellular repair assay**

197 DNA repair capacity can also be measured by including polymerase inhibitors such as
198 aphidicolin or cytosine arabinoside in the cellular repair assay; in this way, removal of the
199 affected nucleotide occurs, but the re-synthesis step to fill the gap in DNA is inhibited [13-15].
200 As a result, the normally transient SBs accumulate to an extent which reflects the repair
201 capacity of the cells. Although from a technical point of view this assay is simple, its application
202 in human biomonitoring studies is very rare. The assay has been successfully used to assess

203 NER capacity [14, 15], but it is worth mentioning that some authors have reported that the
204 DNA breaks produced during NER are not necessarily transient in freshly isolated lymphocytes
205 and are detectable with the comet assay without using additional polymerase inhibitors [16,
206 17]. In any case, the use of polymerase inhibitors may increase the sensitivity of the assay by
207 increasing the %DNA in the tail and avoids misinterpretation of the results (e.g. inter-individual
208 differences resulting from different precursor pool sizes rather than actual differences in
209 repair). The application of this method to biomonitoring requires further investigation and
210 validation.

211

212 **1.2.3 *In vitro* DNA repair assays**

213 As an alternative to assessing repair carried out by intact cells, a more biochemical approach -
214 referred to as *in vitro* DNA repair assay- has been developed. This approach is based on the
215 capability of repair proteins in a cell extract to recognize and incise substrate DNA that
216 contains specific lesions. The whole-cell extract can be prepared from lymphocytes, ground
217 tissues or cultured cells, by ‘snap-freezing’ and subsequent lysis with Triton X-100. At the
218 moment, there are distinct types of *in vitro* DNA repair assay.

219 One of these approaches uses closed circular plasmids containing specific lesions as substrate.
220 When incubated with the cell extract, repair enzymes within this extract can incise the plasmid
221 close to the lesion and the resulting nicked (repaired) or closed (unrepaired) plasmids can be
222 separated by gel electrophoresis [18]. In an alternative version of this plasmid assay, the cell
223 extract is incubated with the plasmid in the presence of ^{32}P-labelled deoxyribonucleoside
224 triphosphates and repair can be estimated by the incorporation of radioactive precursors into
225 a repair patch [19]. In this way the plasmid assay measures the overall repair starting from
226 incision to repair synthesis. Alternatively, the cell extract can be incubated with an
227 oligonucleotide that is constructed with a specific DNA lesion and a terminal radioactive or
228 fluorescent tag [20, 21]. The repair enzymes in the extract will cut the oligonucleotide at the
229 damaged site, causing the release of the label or a change in the size of the single stranded
230 oligonucleotide, which can be measured as an indicator for DNA repair.

231 Although these methods have been applied in human biomonitoring studies, especially by
232 Paz-Elizur *et al*. ([22-24]) and Leitner-Dagan, *et al*. ([25, 26]), the number of studies in which
233 these techniques were applied is limited. The comet assay on the other hand has been used as
234 an *in vitro* DNA repair assay more often and its principle plus multiple applications has recently
235 been reviewed [3, 10]. The DNA incision activity of a cellular extract is measured by incubating

236 it with agarose-embedded nucleoids containing specific lesions as substrate (nucleoids can be
237 derived from established cell lines or white blood cells) (Figure 2). The DNA repair enzymes in
238 the cell extracts will recognize the damage in the substrate nucleoids and induce repair
239 incisions. The comet assay reveals the incision activity of the enzymes by the accumulation of
240 breaks in the substrate nucleoids. (It seems that the pool of deoxynucleotide triphosphates
241 (dNTPs) in peripheral blood mononuclear cells (PBMC) is so limited and diluted that the re-
242 synthesis stage of DNA repair cannot take place. When dNTPs are added to the cellular extract,
243 DNA synthesis and ligation occurs and breaks/incisions are no longer detected [27].

244 Thus, the nature of the DNA lesions in the substrate defines the type of DNA repair that is
245 measured. BER and NER have been extensively studied using this approach [28]. In the case of
246 the *in vitro* BER assay, substrate nucleoids are commonly produced by treating cells with the
247 photosensitiser Ro 19-8022 and subsequent irradiation with visible light or cells are treated
248 with potassium bromate to produce 8-oxoguanine (8-oxoG) [29, 30]. For the *in vitro* NER
249 assay, substrate nucleoids are produced by treating cells with benzo[a]pyrene-diol epoxide
250 (BPDE) to induce bulky adducts [31], with UV(C) to induce pyrimidine dimers [32], or with
251 oxiplatin to induce cross-links [33]. In these *in vitro* assays, the incision activity is considered to
252 be the rate-limiting step of the DNA repair process, and is measured as an indicator of the DNA
253 repair activity. This method is more convenient for human biomonitoring studies than the
254 cellular assay, since several samples can be easily analysed at the same time and it can be
255 performed with frozen samples [34]. Most of the publications using the *in vitro* repair assay to
256 measure DNA repair activity in humans use lymphocytes or PBMCs. There are few human
257 studies using tissue samples; to the best of our knowledge only colon has been analysed [33,
258 35, 36].

259

260 **2. Technical validation and optimizations**

261 The cellular repair comet assay and the *in vitro* repair comet assay for BER and NER have been
262 extensively used in assessing DNA repair for biomonitoring purposes. However, although
263 several protocols regarding the different approaches have been published, most laboratories
264 use their own protocols, which leads to significant variations in procedures and potential
265 difficulties in carrying out inter-laboratory comparisons of results. In fact, all techniques used
266 in molecular epidemiology should be validated before routine use, so that there can be
267 confidence in the results, and comparability between laboratories and studies.

268

269 **2.1. Cellular repair assay – optimizations & lack of validation**

270 Protocols to carry out the cellular repair assay, covering the measurement of repair of SBs,
271 oxidised bases (BER) and UV-induced pyrimidine dimers (NER), were published by Collins and
272 Azqueta [37]. As mentioned in the introduction, this is a simple but tedious approach.
273 According to our knowledge, though the approach has been extensively used, validation
274 studies have not been carried out (or published) and there are still some pending technical
275 issues. Foremost, the effect of the type of DNA-damaging agent on DNA repair activity has not
276 been tested (*e.g*. X-, ɣ-irradiation, *vs*. H_2O_2 to induce SBs; different photosensitisers plus light
277 *vs*. potassium bromate to induce oxidised bases; UVC-light *vs*. BPDE to induce lesions repaired
278 by NER). Additionally, analysis of results is an issue since individuals can vary in the level of
279 damage induced experimentally in the test cells, due to differing individual susceptibility to the
280 DNA-damaging agent (e.g. varying antioxidant status leading to different levels of base
281 oxidation). Therefore, the use of t½ (*i.e*. the period of time at which half of the DNA damage
282 has been repaired) could be a good option in order to compare results among different
283 individuals, assuming first order kinetics, because t½ may be independent of the initial amount
284 of damage [37].

285 The approach of measuring DNA repair by blocking polymerase and re-ligation after incision
286 using DNA polymerase inhibitors has been described as a potential tool to be used in human
287 biomonitoring studies, but it has not yet been applied in large scale studies [15, 38]. It presents
288 the same unsolved technical issues as the cellular repair assay.

289

290 **2.2. The validity of the *in vitro* repair assay**

291 Collins and Azqueta described the practical details for applying the *in vitro* repair assay [37]
292 and a detailed protocol to carry out this assay in cultured cell lines, blood cells, animal tissues
293 and human biopsies, was published in 2013 [34]. The protocol includes practical tips and
294 recommendation for setting up the assays. This is the most convenient adaptation of the
295 comet assay to measure DNA repair in human biomonitoring studies and several technical
296 validations have been carried out.

297 The usefulness of the *in vitro* BER assay was demonstrated several times since the very first
298 paper in which the approach was described, measuring the repair activity of extracts from
299 cells/tissues of OGG1 knockout cells and mice in comparison to wild type (WT) material. In all
300 cases the activity decreased or completely disappeared in knockout samples [29, 36, 39, 40].
301 The usefulness of the *in vitro* repair assay to assess NER activity was demonstrated by Langie *et*

302 *al*. using extracts of cell lines established from patients with xeroderma pigmentosum (XPA-/-,
303 XPC-/-) and WT fibroblasts [31]. They found lower DNA incision activity when extracts from the
304 knockout cells were used and, as expected, the activity was restored to normal WT values
305 when mixing the extracts of XPC and XPA mutants, because they complement each other.
306 Slyskova *et al*. measured the NER repair activity of extract from liver of XPG-/- and WT mice,
307 showing that knockout mice had no more activity than the negative control incubations with
308 reaction buffer alone [36].

309 The protein concentration of extracts can be measured and concentration adjusted, though
310 Collins *et al*. reckoned that determining the extract concentration on basis of the cell numbers
311 is sufficiently accurate when using lymphocytes [29]. However, in some cases cells are lost
312 during centrifugation; and the extraction efficiency of proteins can differ slightly between
313 batches. Therefore, it is recommended that the concentration of proteins should be measured
314 in each extract [34]. In the case of extracts from tissues, the protein estimation is essential [40,
315 41], since tissue samples consist of an unknown number of cells, containing a mixture of cell
316 types and connective tissue.

317 The incision activity at different extract protein concentrations normally shows a non-linear
318 relation or a linear but not proportional relationship between incision and concentration.
319 Collins *et al*., showed a linear but not proportional relationship of extract concentration and
320 BER activity when 0.25X, 0.5X and 1X extract was used [29]. Guarnieri *et al*. also found a linear
321 but non-proportional relationship when testing the BER activity of different mouse liver extract
322 concentrations (0.001, 0.01, 0.1 and 1x) [39]. In an experiment testing different concentrations
323 of extract from human colon biopsies (0-18 mg protein/ml), a non-linear relationship was
324 reported: a non-proportional increase in activity was seen until 3 mg/mL followed by a
325 decrease at higher concentrations [36]. The authors explained that too high protein
326 concentration saturated the reaction. In the same study, similar effects were observed when
327 the NER incision activity was measured. Likewise, when testing human lymphocytes or
328 cultured fibroblast for their NER activity; high protein concentrations caused a lower relative
329 difference between the total damage-related incision activity and non-specific incisions [31].
330 Therefore, when working with tissues, extract dilution curves should be performed to
331 elucidate the protein concentration showing the maximum activity, since important
332 differences in the optimal concentration among tissues (especially between proliferative and
333 non-proliferative tissues) have been shown in animals [40, 41]. This probably also applies when
334 using human tissues.

335 Heat inactivation of extracts from animal tissues demonstrated that the SBs in substrate DNA
336 are produced by enzymes contained in the extract and so the assay is measuring enzyme
337 activity [40-42]. Slyskova *et al*. used aphidicolin or ABT888, inhibitors of the post-incision
338 (repair synthesis) phase of BER and NER respectively, to check if they could increase the
339 specificity of the assay and prevent underestimation of the detected incision activity of the
340 protein extracts (from human colon biopsies) [36]. Incision activity could be underestimated
341 due to the presence of some level of repair synthesis occurring. However, if that were the
342 case, the inhibitors would have enhanced the yield of breaks, but this was not observed [36].

343 The lack of non-specific nucleases in extracts from lymphocytes has been demonstrated by the
344 low level of SBs present in untreated substrates [29]. However, significant non-specific
345 nuclease activity was detected in extracts from animal tissues [40, 41]. In this case, altering the
346 reaction buffer was used as strategy to decrease the non-specific enzyme activity (adding
347 proteinase inhibitors, ATP, polyAT) [40, 41]. Although these changes decreased the non-
348 specific nuclease activity, a simultaneous decrease in repair-specific incision activity was
349 observed. Interestingly, aphidicolin may have an inhibitory effect on various nucleases that are
350 not related to DNA repair processes. For instance it had been demonstrated to inhibit Herpes
351 Simplex virus DNA polymerase-associated nuclease activity [43], as well as the 3'→5'
352 exonuclease activity of eukaryotic polymerases δ and ε [44, 45]. Only the use of aphidicolin
353 significantly increased the specific incision activity of mouse liver and brain extracts by
354 decreasing the non-specific endonuclease activity in the BER assay [40], but did not have such
355 an effect in mouse colon and lung [41]. To reduce non-specific incision activity in mouse colon
356 and lung the protein concentration of extracts had to be decreased or additional washes
357 during extraction had to be performed [41].

358 The reproducibility or inter-experimental variability of the assay has also been demonstrated
359 for different types of samples (lymphocytes and colon biopsies) for both the *in vitro* BER and *in*
360 *vitro* NER assay by analysing duplicate samples on different days [29, 31, 32, 36]. This indicates
361 that the repair activity is stable after storage of samples. Similarly, long-term preservation of
362 animal tissues and extracts to be used in the *in vitro* DNA repair assays has been demonstrated
363 for BER [40]. Regarding the NER assay, the situation depends on the storage of the sample;
364 either as cell pellet or protein extract, plus the addition of ATP to the extract. The use of ATP or
365 an ATP-regenerating system in the extract is not needed when the BER assay is carried out in
366 lymphocytes [29]. When assessing NER incision activity similar results have been obtained with
367 or without adding ATP to freshly prepared cell extracts [31, 32], demonstrating that samples
368 contain enough ATP to carry out the first reactions of the repair process. However, Langie *et*

369 *al*. showed that protein extracts lose their activity after long-term storage (i.e. several weeks)
370 at -80°C and that activity is restored by adding ATP [31]. Cell pellets stored at -20°C kept their
371 activity for at least 40 days and the addition of ATP did not increase activity [31]. Some authors
372 claim that magnesium is essential for the detection of NER activity [32]. However, it is
373 advisable to test this for each new cell type or tissue under study, as a too high magnesium
374 concentration in the extract could enhance non-specific nuclease activity, as demonstrated in
375 the BER assay when using mouse tissue extracts [40, 41].

376

377 **2.3 Crucial parameters to consider**

378 The incubation time of the extract with the substrate is a critical parameter of the assay; time-
379 course experiments showed an initial linear increase in SBs followed by a plateau [29]. The
380 optimal time of incubation should be selected from the linear part of the curve, but showing a
381 high enough BER or NER incision activity. Several incubation times have been reported, many
382 of them based on preliminary studies [31, 39, 40]. These variations in incubation times could
383 be partially due to the different adopted incubation methods; some researchers use humid
384 boxes placed in an incubator, while others use a 'slide moat'. It is crucial to select an
385 incubation time which detects enzyme or extract activity in the linear phase of the titration
386 curve, not to reach the plateau.

387 Langie *et al*., studied the effect of varying the agarose concentration in the BER assay; the
388 agarose concentration may affect the penetration of the enzyme and in consequence the
389 incision repair activity of, in this case, mouse liver extracts [40]. Indeed, an inter-laboratory
390 comparison was published in 2013 [46], in which the incubation step of the nucleoids with the
391 repair extract seemed to be an important stage in the protocol that led to large inter-
392 laboratory variation. In this trial, 8 laboratories tested the BER activity of three cell lines
393 starting with cell pellets or with cell extracts, both provided by the coordinating laboratory.
394 The 6 most experienced laboratories reported the same cell line as having the highest activity.
395 A significant correlation was reported between the repair activity found when testing the
396 provided extract and the self-made extract from the provided cell pellet; this suggests that the
397 predominant source for inter-laboratory variation was the incubation of the extract with the
398 substrate. Though detailed instructions were given to prepare the cell extract or to assess the
399 repair activity of the provided and self-made extracts, each laboratory used their own
400 conditions for the comet assay. Therefore, more attention should be given to standardize this

401 particular steps (i.e., agarose concentration and extract incubation) and the penetration of
402 repair enzymes into the gel.

403

404 **2.4. Outstanding issues that warrant further technical investigation**

405 In the protocol published by Azqueta *et al*., some outstanding technical issues were noted [34].
406 These and additional technical issues are outlined below:

407 1) DNA incision activity can be studied in relation to the number of cells in the extract, the
408 protein concentration or the DNA content, but the accuracy of the different options has not
409 been studied.

410 2) Although, aphidicolin is mainly known as a DNA polymerase inhibitor, the use of aphidicolin
411 in cell extracts also prevents the occurrence of non-specific nuclease activity in the BER assay
412 [40]. Aphidicolin was described to have an inhibitory effect on various nucleases that do not
413 have a specific role in DNA repair processes. However, its effect when the NER assay is carried
414 out has only been tested once with human colon biopsies [36]. In some cases, it may enhance
415 the detection of NER activity by preventing repair synthesis [40].

416 3) There is a lack of proportionality between repair activity and protein concentration, which
417 needs to be further investigated. Meanwhile, it is recommended that as far as possible extracts
418 should be made from the same number of cells or the same wet weight of tissue, and resulting
419 protein concentrations should be checked.

420 4) The *in vitro* repair assay needs to be validated by comparison with other *in vitro* assays.
421 Some efforts have been made in this direction as is stated in the next section.

422 5) A new 'ring study' involving several laboratories, standard cell extracts and standard
423 protocols should be carried out.

424 6) The most widely used substrate for the *in vitro* repair assay has been Ro19-8022 + light.
425 However, potassium bromate is an easier and cheaper chemical to use. This substrate has
426 been used for repair activity in cell cultures [47] and human biomonitoring studies [30].
427 Interestingly, potassium bromate generates equally high levels of DNA lesions detectable in
428 the hOGG1- and Fpg-modified comet assay, whereas Ro19-8022 + light seems to generate
429 lower levels of hOGG1-sensitive sites as compared to Fpg-sensitive sites [48]. This discrepancy
430 remains to be investigated.

431 7) For the NER assay, both UV light and BPDE have been used to produce substrate nucleoids,
432 and the relationship between the two has not been properly studied; cyclobutane pyrimidine

dimers and bulky DNA adducts are not necessarily recognised in the same way by repair enzymes.

8) It has not been studied so far whether the use of different cell types to produce the substrates (e.g. different established cell lines or human lymphocytes) has any influence on the measurement of DNA repair.

9) There is a lack of true positive controls: i.e., compound that increases the cellular repair for the cellular repair assay, or extracts with a high repair activity for the *in vitro* repair assay. This may be a complicated issue since the induction/modulation of the DNA repair may depends on the cells line/tissue under study. However, some attention should be given to this point. Over recent years, different versions of the alkaline comet assay have been developed in order to increase the throughput. For example, a medium throughput comet assay has been successfully used in an updated version of the *in vitro* BER and *in vitro* NER repair assays, using 12 minigels on microscope slides [34, 36, 49], or 8 deposits on GelBond® films for the Aphidicolin-block cellular repair assay [50, 51]. More recently, further adaptations enable high throughput performance of the comet assay. For example, the use of larger Gelbond® films and reduction of the volume of agarose deposited offer the possibility to increase to 96 minigels processed on the same support [52], but this method has not yet been applied to the repair assays. Other technologies derived from the comet assay, using high throughput microarray or microfluidic approaches, have been proposed to study DNA damage, for example CometChip [53], Microfluidic Comet Array [54] and HaloChip [55]. These techniques have been applied to the cellular repair assay, but to date they are not applicable to the *in vitro* repair assay, either for NER or for BER.

High throughput is crucial for human biomonitoring to allow the processing of a high number of samples. A new challenge is to adapt either the high throughput comet assay or one of the newer derived technologies in order to make it useful not only for the cellular repair assay but also for the *in vitro* DNA repair assays.

3. Comparison of techniques and comparison BER/NER

Comparing techniques with each other, preferably comparing a newly developed assay with a gold standard, is a crucial aspect of the validation of a technique, because it provides information about the extent to which the method actually measures the intended outcome (in this case DNA repair activity). Several studies have performed various assays in parallel, but

465 the correlations between the outputs of these assays are rarely described. In this section, we
466 describe the various comparisons that have been investigated to date.

467 A few reports compared data from the comet-based cellular repair assay against plasmid-
468 based repair assays to study BER. Astley et al. (2002) observed an increase in the removal of
469 H_2O_2-induced SBs in carotenoid-supplemented Molt-17 cells by the cellular repair assay, but
470 were unable to confirm these data by means of DNA repair patch plasmid synthesis assays
471 [56].

472 Incubation of H_2O_2-treated HeLa and Caco-2 cells with β-cryptoxanthin, a common carotenoid,
473 led to a ~2-fold increase in the rate of removal of oxidised purines by BER in the cellular repair
474 assay. This effect was confirmed with the *in vitro* BER assay; incision activity was about twice
475 as high with the extract prepared from carotenoid pre-incubated cells [57]. Ramos *et al.* (2010)
476 showed that water extracts from the *Salvia* species *Salvia officinalis* and *Salvia fruticose*, and
477 the polyphenolic compound luteolin-7-glucoside increased the rate of H_2O_2-induced DNA SB
478 removal in Caco-2 cells [58]. Similarly, pre-incubation for 24 h with extracts of *Salvia Officinalis*
479 and luteolin-7-glucoside increased BER-related incision activity in Caco-2 cells. The same group
480 observed the triterpenoid ursolic acid and the flavonoid luteolin (two compounds present in
481 fruits and vegetables) to enhance the H_2O_2-induced SBs removal rate and BER-related incision
482 activity in pre-treated Caco-2 cells [59].

483 Although BER has been studied the most, several studies also use the *in vitro* DNA repair assay
484 to study NER in humans [31, 32, 35, 36, 60-62], as well as in cell lines [63, 64], and in animal
485 models [65, 66]. However, as far as we know, only one study reported a correlation of the *in*
486 *vitro* NER assay with another functional DNA repair method, *i.e.* BPDE–DNA adduct removal
487 over 48 hours as determined by ^{32}P-post-labelling [31]. The slopes of the BPDE-DNA adduct
488 removal curves, were plotted against the DNA incision activity values as measured by the *in*
489 *vitro* NER assay on substrates containing BPDE-DNA lesions, and showed a significant positive
490 correlation between the two assays (linear regression: R^2=0.76).

491 Although NER can act as a back-up mechanism for BER in situations of massive oxidative stress
492 paired with high levels of damaged DNA [67-69], these two repair mechanisms are not always
493 affected in the same way by external factors or disease conditions. In a study of seventy
494 patients with sporadic colorectal cancer, BER and NER activities showed a significant positive
495 correlation in healthy colon epithelium (Pearson test: R=0.32) [35]. In contrast, Gaivao *et al.*
496 (2009) did not observe a statistically significant correlation between BER and NER activity in
497 lymphocytes of healthy volunteers [32]. Still, a direct comparison of NER and BER activity is not

498 necessarily informative, because they recognize and repair different types of DNA lesions. In
499 some cases, NER and BER can even be modulated in opposite directions. For instance, Brevik *et*
500 *al.* (2011) observed that BER and NER activities were affected in the opposite way by kiwi fruit
501 and phytochemical consumption (i.e. high intake of a variety of antioxidant-rich plant
502 products) [62]. In addition, storage of blood samples at room temperature for 24h reduced
503 NER activity as assessed by the aphidicolin-block cellular repair assay for NER compared to
504 fresh samples, whereas OGG1 activity (representing BER) was higher after 24h storage at room
505 temperature *versus* freshly isolated samples [50].

506 Overall, both the cellular repair assay and *in vitro* repair assays have proven to be useful and
507 sensitive for studying the modulation of DNA repair by nutritional factors, environmental
508 exposures and disease state (also see section 4). We are convinced that new comet-based
509 repair assays to study additional repair pathways are bound to come in the near future. It will
510 be of utmost importance to include comparisons with available functional DNA repair assays
511 into their validation process.

512

513 **4. Inter- and intra-individual variation in DNA repair activity**

514 Variations in DNA repair activity at the level of the individual are poorly investigated. However,
515 it is important to understand the sources of variation. There is currently insufficient knowledge
516 to conclude to what extent the repair activity of an individual is determined by genetics, or
517 whether it can be influenced by environmental factors. Moreover, variation between
518 individuals in both BER and NER activities cannot be explained.

519 Gaivao *et al.* measured DNA repair activity on several occasions in the course of a nutritional
520 intervention study, involving 30 healthy subjects [32]. Both BER and NER were assessed by
521 applying the *in vitro* repair assay. As the intervention appeared to have no effect on the DNA
522 repair activity, data from the six blood samplings - at 4-week intervals – were used to examine
523 both inter- and intra-individual variation. In Table 2, the correlation coefficients for all
524 timepoint comparisons are shown, for both BER and NER separately. In 9 of the 15
525 comparisons of BER rates, the correlation was statistically significant, and this was true for 12
526 out of 15 comparisons of NER rates. It is interesting that the correlation coefficients did not
527 decrease as the time between samplings increased. Thus, although there may be unknown
528 factors that affect repair activity from time to time, there is an underlying consistency, in both
529 BER and NER, for a given individual. While there was considerable inter-individual variation in
530 both BER and NER activity between subjects (coefficients of variation: 32% and 59%

531 respectively), the range between highest and lowest activity was substantially higher for NER.
532 Figure 3 shows, as examples, two of the timepoint comparisons for BER and NER. Although it
533 illustrates the relative consistancy of repair rates for individuals, the figure also shows the
534 variety of repair rates between individuals. For BER, most subjects have rates within a 3-fold
535 range; for NER (using UV-exposed substrates in the repair assay), the range is about 7-fold.
536 This is in line with a previously reported 10-fold difference in NER activity using BPDE-exposed
537 substrates in the repair assay [31]. Interestingly both studies [31, 32], reported that some
538 individuals seem to have negligible repair activity. Whether this has any health implications is
539 unclear. It is possible that a low NER rate indicates a reduced intrinsic capacity to deal with UV-
540 induced cyclobutane pyrimidine dimers or bulky adducts, or it could be that individuals with
541 low measured NER activities are not exposed to DNA damage and therefore their repair
542 enzymes are simply not induced.

543 Similarly, in a group of 122 subjects (mean age 24.5 y, range 19-48 y, 39 men and 83 women),
544 inter-individual variation in NER activity assessed by the aphidicolin-block cellular repair assay
545 in response to BPDE, ranged from 0.66 to 26.14 %DNA in tail (mean 7.38 +/- 4.99 %), showing
546 an almost 40-fold difference across the group [51].

547 There are some other publications comparing repair rates between individuals using different
548 techniques (Table 3). These studies highlight considerable inter-individual variability in the
549 capacity to repair DNA. Certain factors, such as age and sex, might affect repair activity and
550 recognising such factors would be necessary for the design of human studies and
551 interpretation of repair data from such a trial. The following sections therefore describe which
552 factors, whether host factors (*e.g.*, age, sex and genetic polymorphisms in DNA repair genes)
553 or environmental/lifestyle factors (*i.e.*, smoking, status, diet and health status), may contribute
554 significantly to this variability.

555

556 **4.1. Host factors**

557 *Age and sex:*

558 Numerous studies have reported a strong positive link between increasing age, DNA damage
559 and defective repair [40, 41, 74-81]. However, to date few human biomonitoring studies using
560 the comet assay have established the relationship between aging and repair activity (table 4).

561 In one study of 375 participants with occupational exposure to asbestos, stone wool and glass
562 fibre, increasing age was associated with increasing DNA BER activity measured by the *in vitro*
563 DNA repair assay using Ro19-8022 with light to induce damage in substrate cells (Correlation

coefficient R=0.1) [69]. In contrast, in a study of 244 men and women (mean age 41.3), neither BER of oxidative damage (*in vitro* repair assay) nor irradiation-induced repair (cellular comet assay) was affected by either age or sex [83]. A cross-sectional study of subjects from Denmark showed an inverse association between age (18 to 83 years) and BER activity (using $KBrO_3$ treated cells as substrate) in PBMCs; the effect was stronger in women as compared to men [30]. However, in a study specifically designed to assess the impact of age on DNA repair activity, Humphreys *et al*., investigated the relationship between age and BER activity measured by the *in vitro* repair comet assay using Ro19-8022 + light damaged substrate cells [82]. BER was investigated in 3 groups of subjects of increasing age [20-35 y (n-40), 63-70 y (n=35) and 75-82 y (n=22)]. Here, the authors found a positive but weak correlation between age and BER rate (r=0.25). However, it should be mentioned that the authors of this paper state that the inclusion criteria were "relaxed" for the oldest group. Consequently, subjects with disease in the oldest group might have biased the results. DNA repair activity was the same in both sexes.

The relationship between age and DNA repair may be further complicated by differences in repair activity in different strata of population studies defined by sex or race. Trzeciak et al. (2008) used a cellular repair assay to study the impact of these factors on repair of γ-radiation-induced DNA damage in PBMCs from four age-matched groups of male and female whites and African-Americans between ages 30 and 64 [84]. They reported a positive association between repair activity and age in white females, but a statistically non-significant decrease in African-American females.

Overall, the available data suggest that, while sex is not a major contributor to inter-individual variation in repair activity, age is a factor that should be taken into account (for example, by ensuring a similar age distribution in control and test groups) - though as yet there is no indication of a major positive or negative effect. Also animal studies have reported conflicting results. There are recent reports that the effect of age on BER activity (*in vitro* repair assay) can be tissue dependent and that the brain seems to be the most vulnerable for a decline in BER activity with age [40-42, 74]. Future human biomonitoring studies should consider studying DNA repair in other available tissues with different cell turnover, in comparison with blood cells (*e.g*. buccal cells, saliva, colon biopsies, etc.). The effect of race on repair activity and its interaction with age is unclear.

Genetics (polymorphisms in DNA repair genes)

597 Data from human biomonitoring studies, using the comet assay to assess the associations
598 between genetic variations in DNA repair genes and repair activity are scarce. Vodicka *et al*.
599 performed a relatively large-scale study [244 healthy subjects, 183 men and 61 women, mean
600 age 41 ± 11 y], specifically designed to investigate the impact of various genotypes (XRCC1,
601 APE1, hOGG1, XPD, XPG, XPC, XRCC3 and NBS1) on NER and BER activities. BER (*in vitro* DNA
602 repair assay) was significantly lower in people homozygous for the GG variant of hOGG1
603 compared with carriers of the normal genotype [83]. The ability to repair γ-irradiation damage
604 (cellular repair assay) was significantly lower in individuals homozygous for the XRCC1 AG
605 genotype. However, in a study by Jensen *et al*., healthy subjects did not show any difference in
606 BER activity (*in vitro* repair assay using Ro19-8022 + light as substrate) associated with the
607 hOGG1 Ser326Cys polymorphism; 49 subjects of each genotype were selected and group-
608 matched from a cross-sectional study of 1019 subjects [85]. Interestingly, there are indications
609 for an interplay between BER and NER, or NER playing a role as a back-up mechanism for BER.
610 For instance, a study on occupational exposure to potential genotoxic agents, observed BER
611 activity (using the *in vitro* DNA repair assay) to be significantly higher in subjects carrying the
612 XPA AA normal genotype compared to the AG and GG variants [69].

613 In addition a few studies have investigated the gene-environment interactions. In a study of 36
614 volunteers recruited to explore the impact of nutrient/gene interactions on NER activity (*in*
615 *vitro* DNA repair assay using BPDE-DNA as substrate), subjects were grouped according to
616 genetic polymorphisms in several NER genes (XPA, XPC, ERCC1, ERCC2, ERCC5, ERCC6, and
617 RAD23B; [60]). Here, NER activity was significantly lower in subjects who carried a relatively
618 large number of "low" NER activity alleles. The XPA G23A gene was the strongest predictor for
619 NER activity, with individuals homozygous for the recessive AA variant of the gene
620 demonstrating 3-fold lower repair activity compared to the normal genotype. Interestingly,
621 this same XPA 23A allele was observed to be associated with lower BER activity (*in vitro* DNA
622 repair assay) in colonic tumour tissues, but not in the adjacent healthy tissue [36]. A recent
623 study investigated the impact of genetic polymorphisms on BER repair activity in 43 patients
624 with recurrent depression disorders and 59 controls without disease [86]. The study included
625 12 polymorphisms in 4 key BER genes (hOGG1, MUTYH, PARP1, and LIG3), which were linked
626 to the cellular repair activity on H_2O_2-induced SBs, but it should be noted that the sample size
627 reported here is small for a study investigating the influence of genotype on disease risk.

628

629 **4.2 Lifestyle factors**

630 *Cigarette smoking*

631 Although, a meta-analysis (evaluating 38 studies) indicated higher levels of DNA damage in
632 smokers versus non-smokers [87], information on the effect of cigarette smoking on DNA
633 repair activity is conflicting. SB re-ligation activity in leukocytes following γ-irradiation (10 Gy)
634 (using the cellular repair assay), was higher in current cigarette smokers (n=17), compared
635 with non or ex-smokers (n=23) [88]. Similarly, SB re-ligation activity following exposure to γ-
636 irradiation (5 Gy) was elevated in smokers (n=80, 1.05 ± 0.81 SSB/10^9 Da) compared with non-
637 smokers (n=134, 0.77 ± 0.62 SSB/10^9 Da) [83]. However, in this study, BER (*in vitro* repair
638 assay) was not affected by smoking. BER measured using the *in vitro* DNA repair assay was
639 significantly lower in poorly nourished male smokers (n=46, mean age 39 y) compared to well-
640 nourished males and females (n=39, mean age 27 y), with mean incision activity 65.9 AU (95%
641 CI 60.4, 70.0) in smokers compared with 86.1 AU (95% CI 76.2, 99.9) in healthy subjects.
642 Moreover, repair data from the cigarette smokers were substantially less variable within the
643 group when compared with the non-smoking participants (range 30-100 AU and 10 -180 AU in
644 the smoking versus the non-smoking subjects respectively). The same authors also studied the
645 effect of smoking in a cohort of workers in a tire plant by performing the cellular repair assay
646 and the *in vitro* BER assay [89]. Higher rates of repair of irradiation-induced DNA damage were
647 detected in smokers versus nonsmokers, but this was not confirmed by the *in vitro* BER assay
648 with Ro19-8022+light generated substrate.

649

650 *Dietary factors*

651 The comet assay has been used widely in human biomonitoring to assess both the impact of
652 whole foods (e.g. fruits and vegetables) and specific nutrients (phytophenols, antioxidants and
653 folic acid) on genomic instability, particularly the impact of diet on DNA SSBs and altered DNA
654 bases (e.g. oxidative, alkylation and misincorporated uracil). In addition, several studies have
655 described how nutrition modifies DNA repair activity (Table 5).

656 After the *in vitro* DNA repair assay came into use in 2001, several researchers started
657 performing it in parallel to the cellular repair assay. Cellular extracts from human lymphocytes
658 showed a markedly higher DNA repair incision activity after a single oral dose of 100 mg
659 CoQ10/day for 1 week compared to controls (~3-fold increase in CoQ10 group) as detected by
660 the *in vitro* BER assay [92]. Similarly, the cellular repair assay, studying the removal of Ro 19-
661 8022 + light induced oxidative lesions, detected a statistically significant ~2-fold higher rate of
662 DNA damage removal in CoQ10 supplemented lymphocytes compared to the control group. In

663 a small randomised cross-over design study, subjects consuming between 1 and 3 kiwi fruits
664 daily for 3 weeks significantly increased *in vitro* BER activity (Ro19-8022+light damaged
665 substrate cells) in PBMCs from male (n=6) and female (n = 8) healthy participants (26-54 y of
666 age) [93]. Volunteers who consumed 3 kiwi fruits each day showed a significantly elevated
667 plasma vitamin C level and substantially enhanced BER activity compared with pre-
668 supplementation levels (>60%). Supplementation also increased the resistance of isolated
669 PBMCs to oxidative damage and was associated with reduced DNA SBs and oxidised base
670 damage (Fpg-sensitive sites). In contrast, there was no correlation between individual BER
671 rates and markers of DNA damage. A significant association between BER activity, assessed by
672 the *in vitro* DNA repair assay (Ro19-8022+ light damaged substrate cells) and antioxidant
673 status was described subsequently, with elevated plasma lutein/zeaxanthin correlating with
674 high BER activity [82]. Supplementation with carotenoids for three weeks, showed enhanced
675 re-ligation of H_2O_2-induced SBs and increased DNA repair patch synthesis activity compared to
676 their initial repair activity before the 3-week intervention [94]. Similarly, supplementing male
677 smokers (n=46, mean age 39) with slow release vitamin C (500 mg/day) and vitamin E (182
678 mg/day) was found to significantly increase BER (*in vitro* repair assay) by approx. 27% (95% CI
679 12 – 41%) after 4 weeks [39]. Inter-individual variation in incision activity was generally
680 consistent within this group (range 30-100 AU). In contrast, feeding healthy subjects (n=43
681 men and women, mean age 27 y) 600 g of fruits and vegetables, or the equivalent levels of
682 antioxidant vitamins and minerals as a supplement for 24 d, did not change BER activity
683 measured by the same group and using the same assay [39]. Inter-individual variability in
684 incision activity was substantial, ranging from less than 10 to more than 180 AU, with a mean
685 of 86.1 AU (95% CI 76.2 - 99.9).

686 In a more recent study, feeding male smokers (45-75 y) a diet high in antioxidant-rich fruits
687 and vegetables (n=33) or 3 kiwifruits per day (n=33) for 8 weeks significantly increased total
688 antioxidant levels (2-fold), plasma vitamin C, β-carotene and tocopherol, compared to the
689 control group (n=34). Also BER activity was increased 40% (n=23) and 29% (n=25) upon
690 antioxidant-rich fruits/vegetable or kiwi consumption, respectively) (measured using the *in*
691 *vitro* DNA repair assay) [62]. Surprisingly, NER activity (*in vitro* repair assay and UVC radiation
692 for substrate), was significantly decreased (39% (n=13) and 38% (n=11); upon antioxidant-rich
693 fruits/vegetable or kiwi consumption, respectively). In contrast, feeding young male smokers
694 steamed broccoli (250mg/day for 10 days) did not alter BER activity (*in vitro* repair assay; [91]).
695 A similar lack of effect of antioxidant supplementation on BER (*in vitro* repair assay) has also
696 been described in 48 young healthy volunteers given 100 µg selenium, 450 µg vitamin A, 90 mg

697 vitamin C and 30 mg vitamin E supplements for 6 weeks [71]. Inter-individual BER activity was
698 substantially different between the volunteers (41-fold). NER (using the *in vitro* repair assay
699 with BPDE-DNA as substrate) was also found to be unaffected by supplementing healthy
700 participants (114 female and 54 male subjects aged between 18 and 45 y) flavonoid-rich
701 blueberry and apple juice (1L/day) for 4 weeks [60]. In this study inter-individual variation,
702 while considerable, was maintained across the two sampling periods (correlation: R=0.69).

703 While the majority of studies report the impact of food or supplements rich in dietary
704 antioxidants on DNA repair activity, a few studies have investigated the impact of other key
705 dietary agents. Low intake of folate is associated with an increased risk of several human
706 cancers, particularly colon cancer [95]. Numerous studies have reported that folate deficiency
707 induces genomic instability and malignant transformation *in vitro*, in animals and in human
708 studies [95]. In a relatively large-scale, randomised double blind-placebo controlled
709 intervention study, participants (n=61, 20-60 y of age, male and female non-smokers and non-
710 supplement users) were given 1.2 mg folic acid daily for 12 weeks to investigate whether
711 enhancing folate status could improve markers of genomic stability, including BER incision
712 activity measured using the *in vitro* DNA repair assay [90]. BER incision activity was similar
713 across both intervention groups prior to supplementation, with a median value in both
714 treatment groups of 63 AU, extending from 34 and 93 AU (2.5 fold range). While there was no
715 association between red cell folate status and BER activity at the start of the study, increasing
716 folate intake resulted in significantly decreased BER in those volunteers with the lowest pre-
717 intervention folate levels, indicating that BER can be modulated by folate status.

718 These studies highlight that diet (and supplement use), has a significant influence on DNA
719 repair activity. The impact of other common nutrients and non-nutrients (such as alcohol and
720 caffeine), as well as other lifestyle factors (including physical activity) on inter-individual
721 variation in DNA repair activity measured using the comet assay remains largely unknown and
722 therefore deserves further attention.

723

724 *Health status*

725 The comet assay has been used widely to determine the relationship between DNA damage
726 (as a marker of genome instability) and various diseases including cancer, vascular disease,
727 diabetes and inflammation. [96] [97] [98] [99]. However, only a few studies to date have
728 investigated the impact of health status (particularly malignant transformation) on NER activity
729 using the comet assay.

730 Palyvoda *et al*., measured NER repair of γ-radiation-induced (2 Gy) DNA SBs in lymphocytes
731 isolated from 44 healthy donors and 38 patients with squamous cell carcinoma of the head
732 and neck (SCCHN), prior to treatment [100]. The cellular repair assay, following a time course
733 of repair post-irradiation (0-180 min), was used to measure endogenous DNA SBs, radiation-
734 induced damage, rate of repair and residual or non-repaired damage in isolated lymphocytes
735 cultured for 24 h prior to treatment. Endogenous DNA SBs was almost 3-fold higher in patients
736 with SCCHN compared with healthy subjects (median 90.3 vs 33.3 AU respectively), with
737 significantly more individuals in the cancer group showing a high level of damage. Overall, NER
738 repair rates were not significantly different between participants with and without cancer, due
739 to substantial variation in measured repair activity across all individuals. However, by
740 stratifying individuals into subjects with high endogenous DNA SBs, high induced DNA damage,
741 low NER rate and high residual DNA damage, a significantly higher proportion of cancer cases
742 displayed this "negative phenotype" compared with healthy participants (39.4% vs. 7.3%
743 respectively). The variation in DNA damage and repair in this study was substantial, making it
744 difficult to draw strong conclusions. It is also important to note that cases and controls were
745 not matched in this study, and that age, sex and cigarette smoking status were markedly
746 different between the two groups. A significant association between cancer incidence and low
747 NER rate was observed in a smaller study of SCCHN cases (n=12) and healthy donors (n=15), in
748 this case matched for age, sex and cigarette smoking [88]. Whole blood was used to measure
749 DNA damage and repair following γ-irradiation (10Gy) using the cellular comet assay, without
750 pre-culture, and assessing percentage tail DNA using computerised image analysis. Here, DNA
751 repair activity was significantly lower in patients with SCCHN cancers relative to controls
752 (46.5% v 36.8% respectively).

753 How other human pathologies and effectors of health and disease, such as low-grade
754 sustained inflammation [101], affect individual variation in DNA repair activity is essentially
755 unidentified and represents a substantial gap in knowledge. In any case, the studies so far
756 reported, have been case-control studies and it is not possible to discern whether a difference
757 in repair activity is a cause or an effect of the disease (or possibly an effect of treatment). What
758 is really needed is a prospective study, *i.e.* a large cohort of healthy subjects whose repair
759 activities are measured and who are then followed up for a long enough period of time for
760 disease to develop and be recorded.

761

762 **4.3. General comment on individual variation in DNA repair**

The studies reviewed above show that age, sex, health status, diet, and other lifestyle factors such as smoking, impact to some extent on DNA repair (BER and NER) activity and contribute substantially to the significant inter-individual variation in repair rates described in numerous human studies. It should also be noted that large assay variation may be interpreted wrongly as inter-individual or intra-individual variation. However, if intra-individual variation (estimated from repeat measurements on different occasions) appears to be at a low level, assay variation can be discounted. There is a need for controlled studies that systematically assess inter-, intra- individual and assay variation in for instance ring-trials. One approach would be similar to the ECVAG ring trials on DNA damage endpoints, in which contributors to the overall variation were assessed in a systematic manner [102-105].

Host factors such as age and sex, and certain anthropometric characteristics such as body mass index can be relatively easily dealt with by carefully matching control and test groups. Adjusting for other factors, such as single nucleotide polymorphisms in DNA repair genes is more difficult, principally due to the requirement for substantially larger numbers of participants to adequately power these biomonitoring studies. Genetic variation in DNA repair genes can also be included in intervention studies as effect modifiers [60]. While specific dietary items obviously have an effect on repair activity, as discussed above and reviewed before [106], it is difficult due to lack of information to estimate the impact of other lifestyle factors such as physical activity, sunlight exposure, drug use and health status on inter-individual variation in DNA repair activity. The advantage of studying DNA repair as phenotypic marker rather than single nucleotide polymorphisms or gene expression is that the latter do not take into account epigenetic and post-transcriptional modifications that can affect the final DNA repair activity.

5. Repair activity kinetics and timing

No studies have specifically assessed DNA repair kinetics in a time-course investigation in humans, using multiple sampling over a short period of time. Assessing DNA repair kinetics is important for selecting optimal sampling times relative to exposure. For instance, if exposure to DNA damaging compounds induces DNA repair, a measurement shortly after exposure will indicate higher levels of repair. On the other hand, after a longer period of time when damage has again decreased due to DNA repair or cell death, such an increase in DNA repair activity may no longer be detectable. Unfortunately, most of the information on DNA repair kinetics originates from biomonitoring studies using the *in vitro* DNA repair assay, assessing DNA repair

796 in samples that have been obtained at a single time-point before, during or after a change in
797 exposure.

798 Only one study has investigated the effect of short-term phytochemical supplementation on
799 repair activity. Intake of green tea was associated with increased BER activity in lymphocytes
800 that were obtained 60 and 120 min after drinking 200 mL of freshly prepared tea [107].
801 Although this study suggests that changes in DNA repair activity after a particular exposure can
802 be very quick (minutes to hours), most studies that investigated dietary interventions actually
803 studied the changes over a period of several days to weeks (see paragraph 4.2). Regarding
804 green tea consumption, 12 weeks of regular green tea consumption indeed significantly
805 increased *in vitro* BER activity toward Ro19-8022 + light generated DNA damage in
806 lymphocytes [108], but the study by Ho *et al*. (2014) [107] suggests that this change could
807 already have been detected at much earlier time points. Time points chosen for sampling in
808 other dietary interventions with *in vitro* BER or NER activity as endpoint vary between 1 to 8
809 weeks [92, 62, 71, 93], with reported washout periods between 1 and 2 weeks [92, 93].
810 Interpretation may become more complex if the intervention is performed in smoking
811 individuals, because smoking by itself may already affect BER or NER activity [39, 91] (see
812 paragraph 4.2).

813 It is a matter of debate how the activity of hOGG1 in human cells is regulated, as the *OGG1*
814 gene may be constitutively expressed [28]. Presence of DNA damage seems logical as an
815 inducer of DNA repair. Indeed, animal studies show that DNA repair can be induced by specific
816 DNA damaging triggers and that alterations in repair activity are relatively quick (within days)
817 [65]. In *in vitro* studies with cell lines, induction of BER or NER can occur within hours [58, 59,
818 101, 109]. The number of investigations in which changes in DNA repair were studied after a
819 specific exposure of humans is limited: A study with controlled exposure to wood smoke,
820 although statistically underpowered, showed a slightly increased *in vitro* BER activity and
821 increased urinary 8-oxoGua (*i.e.* repair product of hOGG1) at 20 h post-exposure [110].
822 Another short-term study reported increased levels of oxidatively damaged DNA and unaltered
823 BER activity in PBMCs after 6 or 24 h controlled exposure to traffic-related air pollution [111].
824 Likewise, oral exposure to nanomaterials showed increased levels of oxidatively damaged DNA
825 in the liver of rats at 24 h post-exposure, whereas the *in vitro* BER activity to Ro19-8022 + light
826 generated DNA substrate cells was unaltered [112].

827 To summarise, BER and NER kinetics have not been well investigated in humans and animals.
828 There are inconsistent reports of altered BER activity after dietary interventions and particle
829 exposure, but sampling times are not frequent enough to draw any conclusions on the time

830 frame in which the changes occur. There are currently too few studies on NER activity to
831 speculate about timing of sampling for assessment of changes in repair activity. From the
832 available literature, it is not possible to suggest an optimal time of sampling in relation to
833 exposure for the assessment of BER and NER activity. Therefore, to improve the applicability of
834 DNA repair measurements in human biomonitoring, it is essential to perform studies in which
835 repair activity is assessed at various time points after exposure/ intervention.

836

837 **6. Surrogate vs. target tissues**

838 PBMCs (frequently referred to as lymphocytes) are extensively used to measure DNA repair
839 activity in human biomonitoring studies. They circulate through the whole body and are
840 regarded as sentinel cells since they can have a relatively long life-span [113]. Moreover, they
841 are easily obtained, available in large numbers and easy to handle and culture if necessary. The
842 purity of the cells fraction is normally not specified and a mixture is probably the most
843 commonly used material. While they are convenient as surrogate cells, circulating blood cells
844 are not the target for carcinogenesis, and the response of these cell types does not necessarily
845 mimic the effect in true target tissue cells. Also, confounding factors (e.g., smoking, diet,
846 medication, air pollution, exercise) should be taken into account [33, 114], because the
847 reaction of surrogate cells in the exposure-outcome relationship may be different in target
848 organ cells. However, using white blood cells is relatively non-invasive and they are the
849 surrogate cells of choice in studies where (as is usually the case) the target tissue is not readily
850 attainable [115].

851 There are only 3 studies with humans in which tissues other than lymphocytes or PBMCs have
852 been used to measure DNA repair activity by the comet assay [33, 35, 36]. In these studies,
853 DNA repair activity was measured in colon biopsies and two of these assessed the correlation
854 between DNA repair activity in tissues and PBMCs.

855 Herrera *et al*. observed that DNA cross-link repair activities of colon tumour epithelial cells and
856 lymphocytes from colon cancer patients (using the *in vitro* repair assay) were not correlated.
857 Thus, lymphocytes were not predictive for the repair ability of the tumour [33]. Slyskova *et al*.
858 found a positive correlation in BER and NER activity between PBMCs and healthy colon tissues,
859 but not between PBMCs and colon tumour tissues [35]. More studies are needed to draw
860 conclusions about the suitability of using lymphocytes or PBMCs to reflect the DNA repair
861 activity of healthy target organs. However, studies in which biopsies of organs from healthy
862 people are included are difficult to perform.

863 Epithelial cells, as specialized components of many organs, have the potential of being an
864 attractive bio-matrix to evaluate the DNA repair activity of individuals. Examples of possible
865 sources of exfoliated epithelial cells in human biomonitoring studies are presented in Table 6.
866 Unfortunately, although there are many studies that use the comet assay to measure DNA
867 damage in buccal, nasal, tear duct, lens and corneal epithelial cells [116], DNA repair activity
868 has never been explored in these biological matrices using the comet assay. Most of these cell
869 types, while not necessarily target cells for carcinogenesis, have the distinct feature of coming
870 into direct contact with various environmental xenobiotics, and so they should provide useful
871 information on the initial response of cells to exposure. Another characteristic of most of the
872 cell types is that they have a rapid turnover; therefore they would only reflect recent events
873 that affected DNA repair. Future studies are needed, addressing the quality and quantity of
874 exfoliated cells that need to be obtained in order to apply the *in vitro* repair assays. Cell
875 recovery should be high enough to make extracts of sufficient volume and protein
876 concentration to apply to substrate cells. Cell counts may be insufficient for buccal cells
877 obtained by mouth rinsing or cheek scrapings (unpublished data). Cell counts are in theory
878 sufficiently high for epithelial cells in urine [117]. Broncho-alveolar lavages [118] and induced
879 sputum [119] also produce a sufficient number of cells, but these are predominantly
880 leukocytes. It should also be noted that repair activity measurements in lavages from the
881 airways are complicated by the fact that respirable toxicants can induce the influx of cells from
882 the blood and the composition of cells in the broncho-alveolar lavage fluid is dependent on the
883 type and stage of pulmonary inflammation. The applicability of epithelial cells for the repair
884 assays needs to be established, as a large proportion of the exfoliated cells may be dead. For
885 small (needle) biopsies an amount of approx. 5 mg of tissue should be enough to make protein
886 extracts [38].

887

888 **7. Discussion and conclusion**

889 The comet assay and its modifications to measure DNA repair activity are frequently used in
890 human biomonitoring studies. However, for the correct interpretation of the data of such
891 biomonitoring studies, validation studies are needed that have to date not been performed in
892 a systematic way. In this manuscript, we have compiled the information that is needed for the
893 validation of the DNA repair comet assays, including intra- and inter-Individual variation, repair
894 kinetics, the use of surrogate tissues, and comparison with other methods.

895 The intra-individual variation over a relatively short period of time (weeks to several months) 896 was reported to be small for both NER and BER, because measurements in the same 897 individuals at two different moments correlated significantly and the slope of the regression 898 line was close to 1.0. This indicates that the measurement of DNA repair activity reflects an 899 individual's intrinsic repair activity.

900 How a low DNA repair activity should be interpreted is an open question; a person can have a 901 low repair activity and may thus have a higher cancer risk, but it is also possible that low DNA 902 repair activity simply reflects the absence of exposure, and thus DNA repair is not needed. 903 Therefore, for proper interpretation of DNA repair activity data, a combined analysis with 904 exposure data and/or other biomarkers (particularly DNA damage) is required.

905 It is important to understand the kinetics of DNA repair after exposure. If DNA repair is 906 measured shortly after a DNA damaging exposure, DNA repair may still be induced. On the 907 other hand, when repair activity is assessed at a later time point relative to the exposure, DNA 908 damage may already be removed and repair is no longer needed. Knowledge about the 909 inducibility of DNA repair is therefore indispensable.

910 The literature is equivocal about the regulation of BER, but NER is likely to be inducible. The 911 different DNA repair pathways are likely to have different modes of regulation. BER often deals 912 with DNA damage induced by endogenously produced DNA reactive compounds. For instance, 913 reactive oxygen species are continuously present (and needed) in the body, but can also lead 914 to oxidised DNA bases. Therefore, these oxidised DNA bases can be considered as physiological 915 DNA lesions and the enzymes involved in BER are thus assumed to be in some way 916 constitutive. In contrast, NER most often deals with damage caused by exogenous agents (i.e., 917 chemicals and radiation), so the enzymes involved in this pathway are probably only 918 synthesized when needed in episodes of increased exposure.

919 This inducibility of NER may also be reflected in the inter-individual variation that is observed 920 in the general population, because the inter-individual variation in NER is reportedly higher 921 than the variation in BER. The inducibility of NER may be related to lifestyle factors in 922 combination with the genetic background. Surprisingly, some healthy subjects seem to have 923 undetectable levels of NER when using comet assay approaches, which could reflect a lack of 924 exposure or a limitation of the comet assay approach. This observation therefore needs 925 confirmation by using other assays. For interpreting NER data, we therefore suggest that these 926 should always be combined with exposure data.

Although the literature suggests that BER activity is less inducible, some studies showed that dietary interventions may still increase BER activity. Induction of repair activity can, of course, occur post-translationally as well as at the level of transcriptional regulation. Therefore, more work is needed to understand the impact of lifestyle, including genetic background, exposure and dietary habits on both BER and NER activity.

Human biomonitoring studies most of the time use leukocytes or PBMCs to assess DNA repair activity. Only a limited number of studies showed a correlation between DNA repair in PBMCs and the target tissue cells, so more work is needed to confirm that repair in blood cells actually reflects the intrinsic repair capacity of internal organs. However, the work that has been published to date looks promising. The total blood cell population (*i.e.*, leukocytes) consists of different cell types including monocytes, lymphocytes and granulocytes. These cell types have differences in life span, concentrations in blood and most probably also different levels of DNA repair. If common diseases, such as a simple cold, affect blood composition, this could change the repair activity that is measured when using total WBC. In that case, differences in DNA repair activity between or within individuals could be related to the percentages of the different cell types in the blood sample. One should keep in mind that isolating blood cell subpopulations automatically requires more work and hands-on time when preparing the samples and this may not always be feasible in large scale biomonitoring studies. Therefore, a more thorough understanding of DNA repair in blood cell subpopulations may guide the decision to use total white blood cells, isolated PBMCs or PBMC subpopulations in human biomonitoring studies.

It is worth to mention that conflicting results observed in some of the studies summarised in this review can be due to the small sample size. However, these studies often show biologically relevant effects and can give important information for larger future studies. More studies with higher samples size are needed.

However, in order to analyze large numbers of samples in a limited amount of time, as is often the case in human biomonitoring, there is a need to develop high throughput approaches; for instance the CometChip is an approach to be explored [120]. Even if the number of samples per run is increased, samples may still be analysed in batches. To avoid batch differences, the comet assay should be further optimized by, for instance, standardizing the preparation of substrate cells, including positive and negative controls, and using assay controls. Although the COST-Action hCOMET (CA15132) may address some of these issues, it will need concerted action by the comet assay community to carry out a full technical and field validation of the

repair comet assay, to reduce inter-assay and inter-laboratory variations, and to ensure the proper comparison and interpretation of results of biomonitoring studies.

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1341 **Legends (figures and tables)**

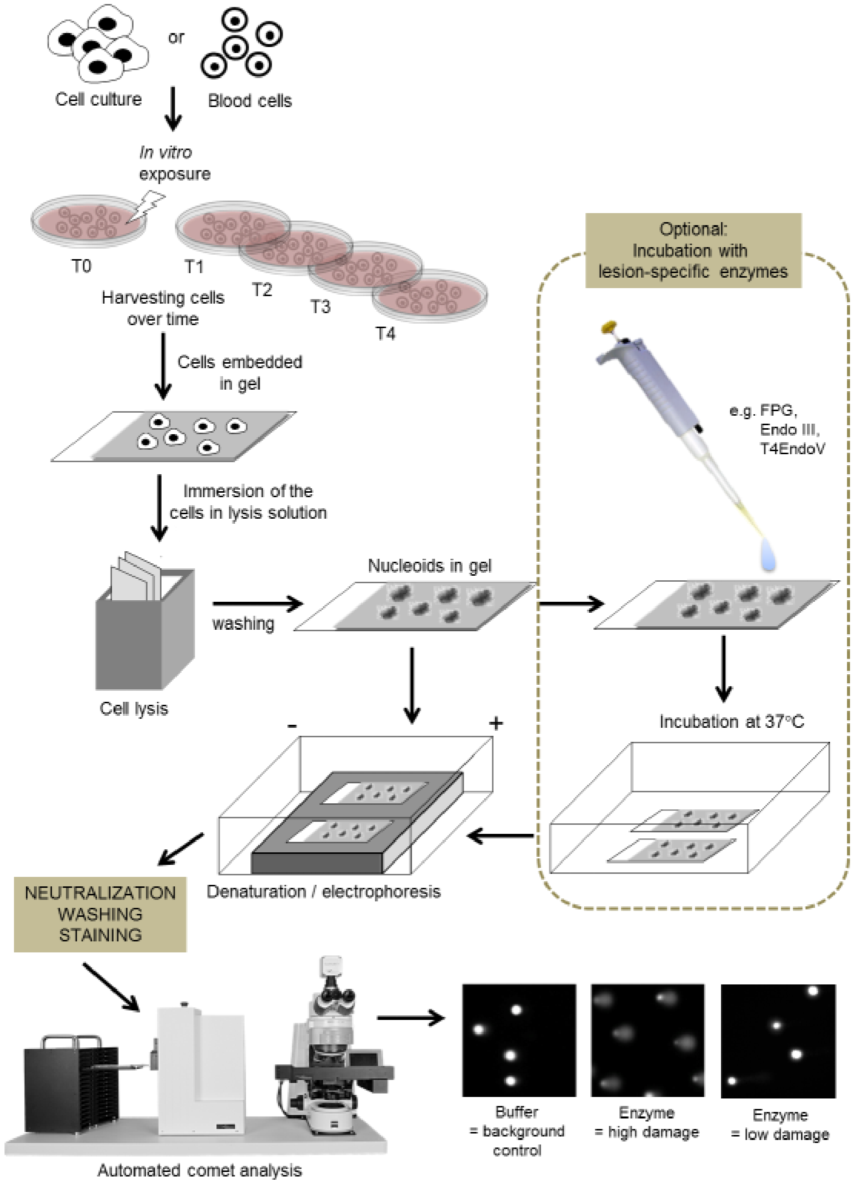
1342

1343 Figure 1: Scheme of the cellular repair assay. Nucleoids can either be incubated with lesion-
1344 specific enzymes (to assess various specific DNA lesions) or not (to assess SBs). The formation
1345 and removal of DNA lesions is studied over time, requiring multiple cell incubations

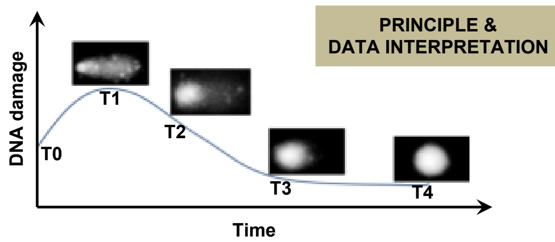
1346

1347 Figure 2: Scheme of the *in vitro* repair assay. Substrate cells can be exposed to the
1348 photosensitizer Ro 19-8022 plus light to induce 8-oxodG lesions or to UV to induce primer
1349 dimers, allowing the assessment of BER and NER incision activity respectively. After lysis, gel-

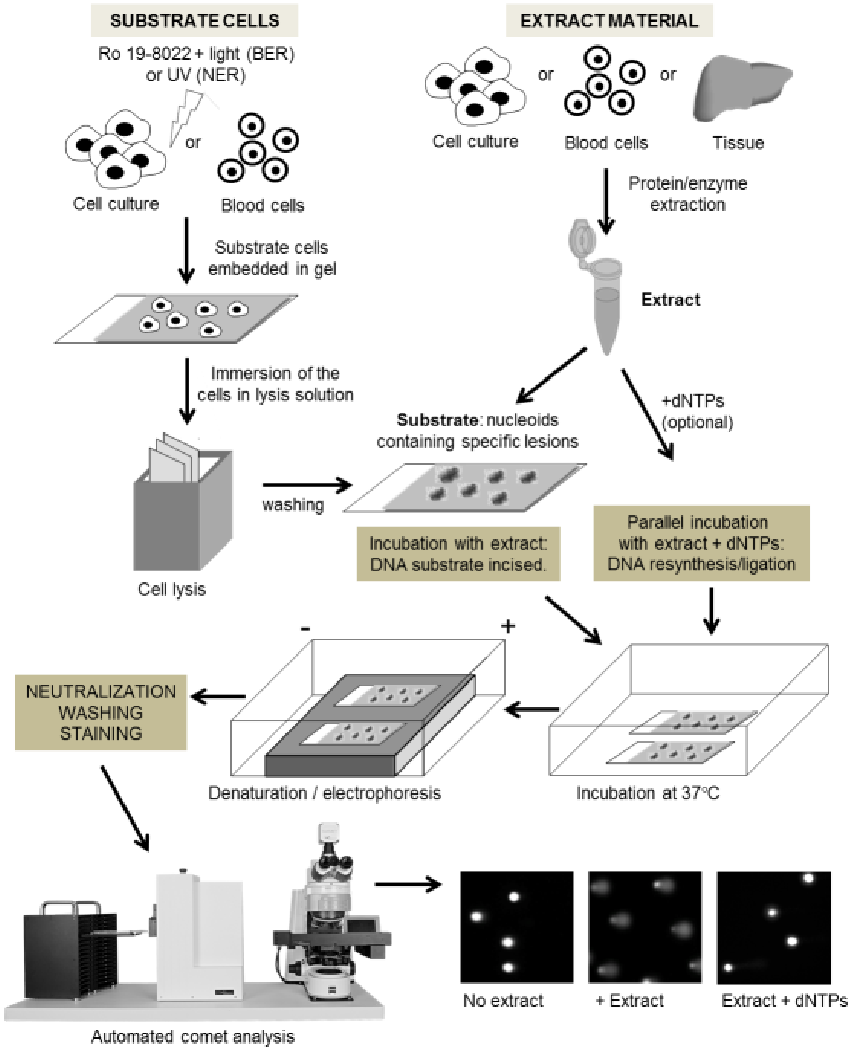
1350 embedded nucleoids are incubated with protein extracts for cells in culture, blood or tissues.
1351 Subsequent standard single-cell gel electrophoresis reveals the SSBs introduced by the DNA
1352 repair enzymes. The addition of dNTPs to the extracts would allow to study DNA
1353 synthesis/ligation capacity in parallel to DNA incision activity.
1354 Figure 3: Correlation between repair activities in extracts from human lymphocytes taken at
1355 different dates (approximately 2 months apart) and analysed using the BER (A) and NER (B) *in*
1356 *vitro* repair assay. Taken from [32], with permission.
1357
1358 Table 1. Overview of human DNA repair mechanims. Taken from [3], with permission.
1359
1360 Table 2. Correlation coefficients, R, for repair rates of 33 individuals at different sampling times
1361 (blood samples were taken approximately 4 weeks apart). * $p<0.05$. Adapted from [32], with
1362 permission.
1363
1364 Table 3. Inter-individual variation in repair activity using different assays. Data from samples
1365 taking at two occasions were available; mean values were used to estimate the range. Adapted
1366 from [12], with permission.
1367
1368 Table 4. Studies on association between age and DNA repair activity in leukocytes,
1369 lymphocytes or peripheral mononuclear blood cells.
1370
1371 Table 5. Studies on association between dietary factors and DNA repair activity in leukocytes,
1372 lymphocytes or peripheral mononuclear blood cells.
1373
1374 Table 6. Sources of exfoliated cells that can be collected in human biomonitoring studies
1375
1376



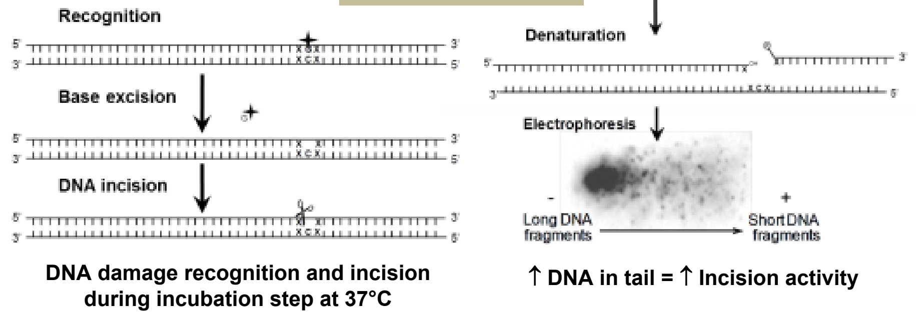
Cell culture
or
Blood cells
In vitro exposure
T0
T1
T2
T3
T4
Harvesting cells over time
Cells embedded in gel
Immersion of the cells in lysis solution
Cell lysis
washing
Nucleoids in gel
Optional: Incubation with lesion-specific enzymes
e.g. FPG, Endo III, T4EndoV
Incubation at 37°C
-
+
Denaturation / electrophoresis
NEUTRALIZATION
WASHING
STAINING
Automated comet analysis
Buffer = background control
Enzyme = high damage
Enzyme = low damage
PRINCIPLE & DATA INTERPRETATION



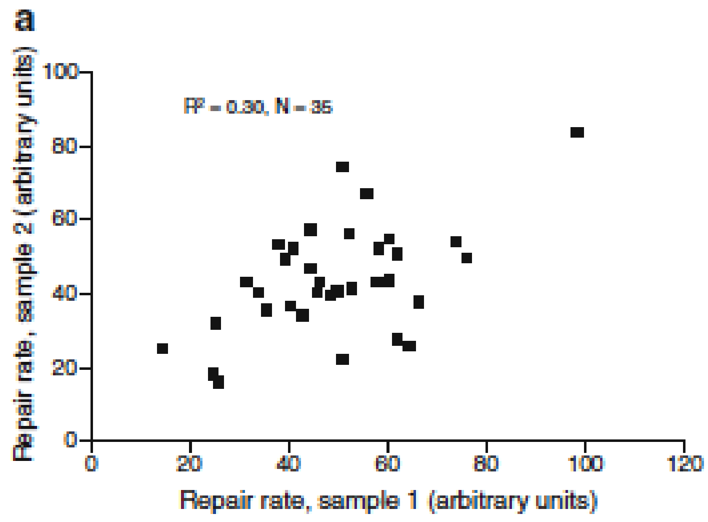
DNA damage
T0
T1
T2
T3
T4
Time

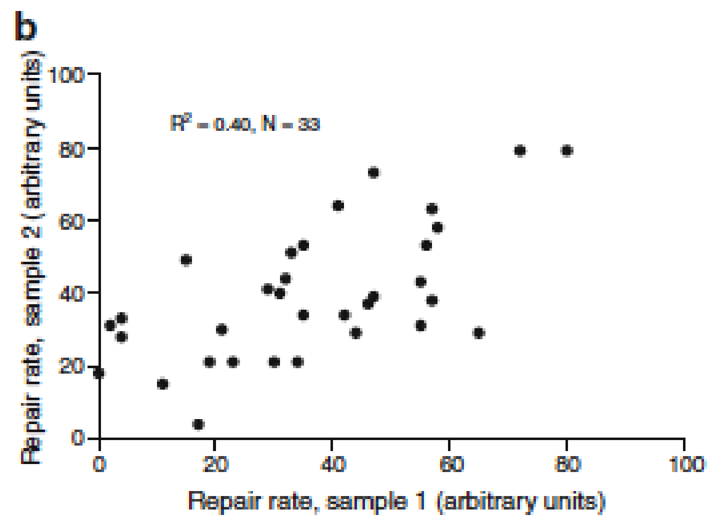


SUBSTRATE CELLS
Ro 19-8022 + light (BER) or UV (NER)
or
Cell culture
Blood cells
Substrate cells embedded in gel
Immersion of the cells in lysis solution
Cell lysis
washing
EXTRACT MATERIAL
or
or
Cell culture
Blood cells
Tissue
Protein/enzyme extraction
Extract
+dNTPs (optional)
Substrate: nucleoids containing specific lesions
Incubation with extract: DNA substrate incised.
Parallel incubation with extract + dNTPs: DNA resynthesis/ligation
-
+
NEUTRALIZATION
WASHING
STAINING
Denaturation / electrophoresis
Incubation at 37°C
Automated comet analysis
No extract
+ Extract
Extract + dNTPs



PRINCIPLE &
DATA INTERPRETATION
Recognition
Base excision
DNA incision
DNA damage recognition and incision during incubation step at 37°C
Denaturation
Electrophoresis
-
+
Long DNA fragments
Short DNA fragments
↑ DNA in tail = ↑ Incision activity


a
Repair rate, sample 2 (arbitrary units)
R^2 = 0.30, N = 35
Repair rate, sample 1 (arbitrary units)
0
20
40
60
80
100
120


b
Repair rate, sample 2 (arbitrary units)
R^2 = 0.40, N = 33
Repair rate, sample 1 (arbitrary units)
0
20
40
60
80
100

Repair pathway	Damage repaired	Sources of damage
Direct reversal	Alkylated base O^6-methyl-guanine, pyrimidine dimers (by photolyase)	Alkylating agents, nitrosoureas, streptozotocin, UV(C) light
Base excision repair	Oxidised bases, alkylated bases, abasic/apurinic/apyrimidinic sites, single-strand breaks	Reactive oxygen species (ROS), alkylating agents, ionizing radiation, spontaneous hydrolysis
Nucleotide excision repair	Bulky helix-distorting lesions, intra-strand cross links, DNA-protein cross links, inter-strand cross links	UV(C) light, cigarette smoke, dietary factors (aflatoxin, poly-aromatic hydrocarbons (benzo(a)pyrene))
Mismatch repair	Mismatched base pairs, small insertion loops	Replication errors, minor base modifications (oxidation, alkylation)
Double-strand break repair (i.e., homologous recombination and non-homologous end-joining)	Double-strand breaks	Ionising radiation, replication errors

	T=0	T ≈ 4 weeks	T ≈ 8 weeks	T ≈ 12 weeks	T ≈ 16 weeks
BER					
T ≈ 4 weeks	0.25				
T ≈ 8 weeks	0.42*	0.11			
T ≈ 12 weeks	0.40*	0.50*	0.30		
T ≈ 16 weeks	0.60*	0.08	0.62*	0.38*	
T ≈ 20 weeks	0.50*	0.35*	0.12	0.32	0.47*
NER					
T ≈ 4 weeks	0.40*				
T ≈ 8 weeks	0.32	0.60*			
T ≈ 12 weeks	0.51*	0.64*	0.52*		
T ≈ 16 weeks	0.44*	0.54*	0.48*	0.40*	
T ≈ 20 weeks	0.45*	0.42*	0.18	0.31	0.59*

Lesion repaired	Assay	N	Range (fold)	Source of variation	Reference
AP-sites	Plasmid	10	2.5	*Healthy individuals (age: 25-48 years). Authors did not correlate repair with other parameters.*	[18]
8-OxoG	Oligonucleotide	34	2	*Healthy individual (age: 18-60). There was no difference in OGG1 activity due to gender and smoking behaviour. Authors did not report age effect. OGG1 polymorphism not associated with altered OGG1 activity.*	[70]
8-OxoG	Oligonucleotide	120	2.8	*Healthy individuals. No significant differences between males and females, or between smokers and non-smokers. OGG1 activity was significantly lower in males older than 55 years compared to younger subjects. This effect was not observed in females.*	[24]
8-OxoG	In vitro comet assay	35	3	*Healthy individuals from intervention study (no effect of the intervention on DNA repair capacity). No further data on individuals' characteristics reported. Authors did not correlate repair with other parameters.*	[32]
8-OxoG	In vitro comet assay	40	41	*Individuals from 18 to 30 years old. Association between endogenous SBs and BER was not observed. Authors did not correlate repair with other parameters..*	[71]
UV-induced damage	Host cell reactivation assay (HCRA): catalase and luciferase assay	102	4.7 (luciferase assay) 7 (catalase assay)	*Healthy subjects (age: 19-79). Authors did analyze correlation with age or other factors.*	[72]
UV-induced damage	In vitro comet assay	33	7	*Healthy individuals from intervention study (no effect of the intervention on DNA repair capacity). No further data on individuals' characteristics reported. Authors did not correlate repair with other parameters.*	[32]
UV-induced damage	Host cell reactivation	63	11	*Individuals from an intervention study (age: 18-30, no effect of the intervention on DNA repair capacity). NER capacity was inversely associated with age, endogenous DNA SBs and BMI (adiposity).*	[73]
Benzo(a)pyrene	In vitro comet assay	8	10	*Healthy individuals; no further data. Authors did not correlate repair with other parameters.*	[31]
BPDE- induced damage	Aphidicoline-block cellular comet assay	122	40	*Healthy people (age: 19-48, cropreserved lymphocytes). Authors did not correlate repair with other parameters.*	[51]

Country (age) number of females (F) and males (M)	Cell type	Comet repair assay (substrate)	Effect on DNA repair biomarker	Adjustment	Reference
Slovakia (21-88 years, 227 M, 161 F) recruited from factories with occupational exposure to asbestos, stone wool or glass fibres and controls matched for age, sex, alcohol consumption and smoking	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells, 10 min incubation)	Positive association between age and repair incision activity (r = 0.1, P<0.05, correlation analysis not specified)	No control for confounding (with regard to age-dependent effects on DNA repair incision activity)	[69]
UK (young (20-35 years), middle (63-70 years) or old (75-82 years), n = 97, MF). Sampled from areas near Bristol, London, Wisbech, Aberdeen and Dundee	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells 10 min incubation)	Positive association between age and repair incision activity (r = 0.25, P = 0.06, Pearson correlation). Statistically significant group difference when tested by ANOVA	No control for confounding	[82]
Czech Republic (41 ± 11 years, 183 M, 61 F) recruited in local administration, medical centres and various branches of plastic industry	Lymphocytes (fresh for repair of DNA strand breaks, frozen for *in vitro* repair assay)	*In vitro* repair (Ro19-8022 + light, Hela cells, 10 min incubation) Repair of gamma radiation induced DNA strand breaks (5 Gy)	No association between age and repair incision activity and gamma radiation-induced DNA strand breaks (results not shown, type of control for confounding not specified in detail)	Analysed by both simple and multivariate regression analysis	[83]
USA (30-64 years, 48F, 48M) of White and African America race	PBMCs (frozen)	Repair of gamma radiation induced DNA strand breaks (6.3 Gy)	Positive association between age and repair activity in White females (r = 0.55, P < 0.01) and borderline statistical significance (r = -0.40, P = 0.06, linear regression) in African-American females. No effect in White or African-American males	Matched in sex and race strata	[84]

Denmark (18-83 years, 40 M, 38 F) from a national health survey in Copenhagen	PBMCs (frozen)	*In vitro* repair ($KBrO_3$, THP-1 cells 45 min incubation)	Inverse association between age and repair incision activity in women, but not in men. Decline in repair activity per year was 0.65% per year (95% CI: 0.16% – 1.14%) in multivariate regression analysis	Sex, body mass index (or waist-hip ratio), blood pressure, cholesterol, triglycerides, Hb1Ac, C-reactive protein, smoking and alcohol	[30]

Country (age) number of females (F) and males (M)	Cell type	Comet repair assay (substrate)	Effect on DNA repair biomarker	Effect on phytochemical	Reference
Sequential study of non-smokers (20-50 years, n = 6, M, Scotland) ingesting 100 mg/day of CoQ_{10} for 1 week and a subsequent washout period of 1 week	Lymphocytes (fresh)	*In vitro* repair (Ro19-8022 + light, lymphocytes, 20 min incubation)	Increased repair incision activity after supplementation compared to pre-supplementation. Decreased levels compared to supplementation period, although not statistically significant, after 1 week washout period	Increased CoQ_{10} concentration in plasma	[92]
Cross-over study on healthy non-smoking subjects (26-54 years, n = 14, MF, Scotland) ingesting 1, 2 or 3 kiwifruits/day for 3 weeks	Lymphocytes (fresh)	*In vitro* repair (Ro19-8022 + light, Hela cells, 10 min incubation)	Increased repair incision activity after consumption of kiwifruits (similar effect of 1-3 kiwifruits/day)	Increased plasma concentration of vitamin C concentration	[93]
Placebo-controlled parallel trial on non-smokers (18-50 years, n = 20, UK) ingesting tablets with α-carotene (3.7 mg) and β-carotene (8.2 mg) for 3 weeks	Lymphocytes (fresh)	Repair of H_2O_2 induced DNA strand breaks (100 µM)	DNA repair of DNA strand breaks over a 4 h incubation period (no repair in cells from the placebo group). Groups of subjects with intake of cooked carrots, mandarin oranges and vitamin C tablets were included in the study, but the results are not reported (risk of reporting bias)	Increased plasma concentration of β-carotene	[94]
Placebo-controlled parallel trial on non-smokers (20-60 years, n = 61, MF, UK) ingesting folic acid (1.6 mg/day) for 12 weeks	Lymphocytes (not specified)	*In vitro* repair (Ro19-8022 + light, CHO cells 20 min incubation)	Unaltered levels of repair incision activity in the whole study population. A restricted analysis of the quartile with lowest baseline red cell folate concentration showed a reduction of repair incision activity in the folate	Increased 5-methyltetrahydrofolate concentration in plasma, erythrocytes, and lymphocytes	[90]

			supplementation group (risk of bias due to subgroup analysis and unequal baseline folate concentration between supplementation and placebo group)		
Cross-sectional study of (young (20-35 years), middle (63-70 years) or old (75-82 years), n = 97, MF) from areas near Bristol, London, Wisbech, Aberdeen and Dundee	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells 10 min incubation)	Inverse correlation between plasma concentration of lutein/zeaxanthin (r = -0.31, P = 0.06, Pearson correlation). Marginally positive association with retinol (r = 0.25, P = 0.06). No correlation with vitamin C, β-carotene, lycopene and α-tocopherol. No control for confounding	Not applicable	[82]
Placebo-controlled parallel trial on smokers (39 ± 12 years, n = 48, M, Denmark) ingesting 500 mg vitamin C and 182 mg vitamin E per day for 4 weeks	PBMCs (frozen)	*In vitro* repair (Ro19-8022 + light, A549 cells, 20 min incubation)	Increased repair incision activity in the group of subjects who ingested vitamin C and E as slow-release tablets. No effect in the group that received tablets with fast-release tablets	Increased vitamin C in plasma after ingestion of both slow- and fast-release tablets	[39]
Placebo-controlled parallel trial on non-smokers (27 ± 6 years, n = 43, MF, Denmark) ingesting 600 g fruit/vegetables or tablets with the corresponding amount of vitamins and minerals for 4 weeks	PBMCs (frozen)	*In vitro* repair (Ro19-8022 + light, A549 cells, 20 min incubation)	Unaltered levels of repair incision activity	Strong decrease in plasma vitamin C in the placebo group. Increased lycopene levels (fruit/vegetable group) and β-carotene (tablet group)	[39]
Sequential study of non-smokers (18-45 years, n =	Lymphocytes (frozen)	*In vitro* repair (benzo[a]pyrene-	Unaltered levels of repair incision activity	Not reported	[60]

36, MF, Netherlands), selected according to ERCC1 genotype, ingesting blueberry and apple juice for 4 weeks after a 5-day washout period		diolepoxide, cells or incubation not reported)			
Placebo-controlled parallel trial on non-smokers (18-30 years, n = 48, MF, UK) ingesting a supplement (100 µg Selenium, 450 µg vitamin A, 450 µg retinol, 90 mg vitamin C and 30 mg/ vitamin E) for 6 weeks	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells, 20 min incubation)	Unaltered levels of repair incision activity	Not reported	[71]
Cross-over study on smokers (22 ± 3 years, sex not specified, Italy) ingesting steamed broccoli (250 g/day) for 10 days	PBMCs (frozen)	*In vitro* repair (Ro19-8022 + light, A549 cells, 20 min incubation)	Unaltered levels of repair incision activity	Increased plasma concentration of folate and lutein. Unaltered levels of β-carotene	[91]
Placebo-controlled parallel trial on smokers (45-75 years, n = 69, M, Norway) ingesting kiwifruit or a phytochemical-rich diet for 8 weeks	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, substrate cell not reported, 20 min incubation; UV-C, substrate cell not reported, 30 min)	Increased base excision (Ro19-8022 + light) and decreased nucleotide excision (UV-C) repair in both kiwifruit and phytochemical-rich diet group	Increased vitamin C (both groups). Increased β-carotene and tocopherol in the phytochemical-rich group	[62]

Target tissue	Surrogate tissue	How to obtain cells
Bladder	Exfoliated epithelial cells	• Isolate from urine
Upper respiratory tract	Buccal cells Nasal epithelial cells Mouth cells	• Mouth rinse or scraping • Nasal lavage • Isolate from saliva
Lower respiratory tract	Lung derived cells	• Isolate cells from induced or spontaneously produced sputum • Broncho-alveolar lavage
Colon	Exfoliated epithelial cells	• Isolate from stool
Mammary	Exfoliated epithelial cells	Isolate from • Nipple aspirate • Ductal lavage • Breast milk
Prostate / testis	Epithelial cells spermatozoa	• Isolate from ejaculate
Other tissues		• Biopsy (invasive)