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Different aspects of cytochalasin B Blocked micronucleus cytome (CBMN cyt) assay as a comprehensive measurement tool for radiobiological studies, biological dosimetry and genome instability

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► ***Review article***

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ABSTRACT

It is now universally accepted that DNA is the main target for damages caused by physical and chemical genotoxicants. Although there are different methods to measure directly the induced DNA damages but due to fast repair processes in cellular environment, most of the damages would be repaired even before sampling, therefore processed DNA damages, i.e. damages left unrepaired after acting repair machinery is preferable to measure in various exposure scenarios. Various cytogenetic end points are introduced and implemented such as metaphase analysis, sister chromatid exchanges, premature chromosome condensation, translocation assay and micronucleus assay. All of these methods were extensively used for various purposes but among them micronucleus (MN) assay was found more practical because of ease of scoring, potential for automation as well as being nearly as sensitive as other procedures. These characteristics made MN assay very popular for screening of the effects of various genotoxic agents in vitro and in vivo. In this review we try to summarize the main aspects of application of this method in radiobiological studies and genome instability related diseases.

Keywords: *Cytome assay, micronucleus, genome instability, radiobiological studies, biological dosim.*

INTRODUCTION

Ionizing radiations as well as a vast number of chemical agents are capable of inducing DNA damage directly or indirectly via formation of free radicals and oxidative agents. The most important lesions induced in DNA include base damage (bd), single strand break (ssb), double strand break (dsb), DNA-DNA cross link and DNA-protein cross link. All DNA damages undergo various DNA repair processes and unrepaired DNA damage lead to genome instability of cell and formation of chromosomal abnormalities. The origin of chromosomal aberrations is not fully understood but there are evidences showing DNA dsb as major cause of chromosomal aberration formation. There are various cytogenetic methods such as metaphase analysis, sister chromatid exchanges, premature chromosome condensation and micronuclei assay for assessment of genotoxicity induced in cells expressed as chromosomal abnormalities. Among these methods micronuclei assay got greater attention because of ease of the scoring and potential for automation. In vitro micronuclei assay was first performed by Heddle [(1)] on mono-nuclear lymphocytes. Later in 1985 when Fenech introduced cytochalasin-B blocked micronucleus assay, where presence of micronucleus is scored in bi-nucleate lymphocytes allowing observation of chromosomal damage after the first division of cells, made this method more popular than before [(2)].

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As mentioned earlier The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring micronucleus (MN) in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided Bi-nucleated (BN) cells, which are the cells that can express MN [2,3]. In the CBMN assay, once-divided cells are recognized by their BN appearance after blocking cytokinesis with cytochalasin-B (Cyt-B), an inhibitor of microfilament ring assembly required for the completion of cytokinesis [2,3]. Restricting scoring of MN in BN cells prevents confounding effects caused by suboptimal or altered cell division kinetics, which is a major variable in micronucleus (MN) assay protocols that do not distinguish between non-dividing cells that cannot express MN and dividing cells that can [2,3]. Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests and an established comprehensive method for assessing cytostasis and chromosome stability, chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis or genetic toxicology and radiation sensitivity testing in human and mammalian cells [4-16]. The CBMN assay is a multi-endpoint assay that measures not only chromosome damage [i.e., micronuclei reflecting chromosome breaks; nucleoplasmic bridges (NPB) reflecting chromosome rearrangements; and nuclear buds (NBUD) reflecting gene amplification] but also other cellular events (such as apoptosis and necrosis). It was reported that the base-line frequencies of MN frequency varied greatly between populations also age and gender were the two most important demographic factors influencing MN frequency. The scoring criteria used were probably the most important method variable influencing the score obtained [17]. Compared with other cytogenetic assays, quantification of micronuclei confers several advantages, including speed and ease of analysis, no requirement for metaphase cells and reliable identification of cells that have completed only 1 nuclear division. This prevents confounding effects caused by differences in cell division kinetics because expression of the micronuclei is dependent on completion of nuclear division [18]. The wide-spread use of the CBMN assay has made it feasible to launch an international collaborative project, the HUMN project [19], with the following objectives: determining the important methodological, demographic and life-style variables that influence this index of DNA damage, establishing standardized protocols for the assay, and ultimately to determine the capacity of this biomarker to predict cancer risk, as well as the risk of other degenerative diseases. The term CBMN Cyt is now used because the original cytokinesis-block micronucleus (CBMN) assay was restricted to measuring micronucleus (MN) frequency in binucleated cells but has since evolved into a comprehensive "cytome" system for measuring DNA damage, cytostasis, and cytotoxicity. The ''cytome'' concept implies that every cell in the system studied is scored cytologically for its viability status (necrosis, apoptosis), its mitotic status (mononucleated, BN, multinucleated) and its chromosomal damage or instability status (presence of MN, NPBs, NBUDs and number of centromere probe signals among nuclei or MN of BN cell if such molecular tools are used in combination with the assay). For these reasons, it is now appropriate to refer to this technique as the cytokinesis block MN cytome (CBMN Cyt) assay. The protocol for which has been previously detailed by Fenech [18]. In brief, DNA damage events are scored specifically in once-divided binucleated (BN) cells and include micronuclei (MN) which is a biomarker of chromosome breakage and/or whole chromosome loss, nucleoplasmic bridges (NPBs), The nuclear buds (NBuds) also the Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios [18]. In nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end fusions, typically, a dicentric chromosome and an acentric chromosome fragment are formed that result in the formation of an NPB and an MN, respectively. Misrepair of DNA strand breaks could also lead to the formation of dicentric ring chromosomes and concatenated ring chromosomes which could also result in the formation of NPBs [20]. As mentioned an alternative mecha-

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nism for dicentric chromosome and NPB formation is telomere end fusion caused by telomere shortening, loss of telomere capping proteins or defects in telomere cohesion [21], but in this case the NPB is not necessarily accompanied by an acentric chromosome fragment or an MN. NPBs occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. Rarely is it possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis, which ultimately results in breakage of the NPB when the daughter cells separate [22]. However, in the CBMN assay, BN cells with NPBs are allowed to accumulate because cytokinesis is inhibited and the nuclear membrane is eventually formed around the chromosomes allowing an anaphase bridge to be observed as an NPB. Therefore, some MN may also originate from broken anaphase bridges although whether this actually happens in cytokinesis blocked cells remains unclear. The importance of scoring NPBs should not be underestimated because it provides direct evidence of genome damage resulting from misrepaired DNA breaks or telomere end fusions, which is otherwise not possible to deduce by scoring MN only, which could originate from either acentric chromosome fragments or chromosome loss [23]. The nuclear buds (NBuds), a biomarker of elimination of amplified DNA and/ or DNA repair complexes has been observed in cultures grown under strong selective conditions that induce gene amplification as well as under moderate folic acid deficiency [24-28]. In vitro experiments with mammalian cells showed that amplified DNA is selectively localized to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MN during S phase of mitosis. Amplified DNA may be eliminated through recombination between homologous regions within amplified sequences forming mini -circles of acentric and a telomeric DNA (double minutes), which localize to distinct regions within the nucleus, or through the excision of amplified sequences after segregation to distinct regions of the nucleus. The process of nuclear budding occurs during S phase and the NBUDs are characterized by having the same morphology as an MN with the exception that they are linked to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process. The duration of the nuclear budding process and the extrusion of the resulting MN from the cell remain largely unknown [23]. Using methods that can detect centromeric regions of chromosomes helps to take full advantage of the CBMN Cyt assay and obtain deeper understanding of the genotoxic mechanism. This is best achieved by distinguishing between MN originating from whole chromosomes and those originating from acentric fragments as well as to determine whether malsegregation of chromosomes is occurring between nuclei in a BN cell that may or may not contain MN [18]. The use of MN size as a discriminant is not recommended for human cells or other cell types in which the size of chromosomes is heterogenous because a small MN may contain either a fragment of a large chromosome or a whole small chromosome. Centromeric regions of chromosomes can be detected indirectly using antibodies to the kinetochore proteins that are assembled on centromeres but this approach does not distinguish between unique chromosomes and may not detect chromosome loss occurring due to absence of kinetochores on defective centromeres [18]. The preferred method is to use in situ hybridization (ISH) to identify centromeric regions using DNA or peptide nucleic acid pancentromeric probes, which allows MN containing whole chromosomes to be reliably identified. Furthermore, use of centromeric probes that are specific for a unique chromosome can be used to detect non-disjunctional events (i.e., unequal distribution of unique homologous chromosome pairs in daughter nuclei) in BN cells [29]. It is also possible to use a combination of pantelomeric pancentromeric probes to determine the mechanism of MN and NPB formation cells [30-32]. The significance of these developments and the concept of the CBMN assay as a ‘‘cytome’’ assay of chromosomal instability in different fields are explained in the following sections.

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Value of CBMN Cyt assay in radiobiological studies:

In radiobiological studies, CBMN Cyt assay was used as a sensitive tool to measure variety of phenomena such as Radiosensitivity, Radioadaptation, Radioprotection, bystander effect and radiation biodosimetry. There are some evidences as follow:

Radio sensitivity

Radiosensitivity is the relative susceptibleness of cells, tissues, organs or organisms to the dangerous effect of ionizing radiation. Many biological factors affect radiosensitivity. Inherent characteristic is one of the important reasons of differences in radiation sensitivity [33]. The physical specifications of ionizing radiation such as its type (particle or photon), energy and dose rate could alter the biological response of organ or tissue to ionizing radiation. People with higher radiosensitivity, most likely will suffer from deterministic and stochastic effects in radiotherapy [34]. In variety of studies the cytokinesis-block micronucleus assay (CBMN) was used as a tool to measure radiosensitivity [35-37].

Radioadaptation

There is a large body of evidence that radiation has a stimulating effect on a number of biological processes and can induce resistance, a phenomenon generally termed as 'adaptive response'. CBMN assay was used as a cytogenetic technique to show these phenomena in variety of studies [8, 38-41].

Radioprotection

Cytokinesis block micronucleus assay was used in variety of studies to evaluate the radio protective effect of different substances by assess cellular DNA damage [42- 46]. It is well known that exposure to certain environmental substances can cause genetic mutations. Because mutations are often associated with the development of cancer and teratogenesis, information regarding the mutagenic potential of various agents, especially those used in medicine, is crucial [47]. For example effective radiotherapy to treat cancer patients should include maximal tumor cell killing with minimal injury to normal tissue. A cautious balance between the total dose of radiation delivered and the threshold limit of the surrounding normal critical tissues is essential to optimize outcomes. The prevention or treatment of early and late radiotherapy effects would improve quality of life and increase cancer curability by intensifying therapies. In this sense, the role of radio protective compounds is of utmost importance in clinical radiotherapy [48].

One way to prevent DNA damage and potential disease development is to avoid exposure to mutagenic agents. Alternatively, the administration of chemoprotective agents may increase resistance to mutagens/carcinogens and/or inhibit disease progression [48,49].

Antioxidants are substances that can bind free radicals and significantly reduce or prevent oxidation of the substrate. Specifically, antioxidants protect against free radical damage by preventing free radicals from attacking lipids, protein amino acids, and the double bond of polyunsaturated fatty acids and DNA bases, thereby preventing cellular damage [47].

Bystander effect

One of the major paradigm shifts in the field of radiation biology was the discovery of non-targeted effects such as the bystander effect in which cells in the vicinity of radiation-damaged cells behave as though they were also irradiated. The suitability of the CBMN Cyt assay to detect the bystander effect of IR exposure has also been demonstrated in a number of studies using different models, ranging from single cell layer models with/without inhibitors of gap junctions [50] to 3-D skin models in which it was shown that MN and apoptosis induction was highest in the vicinity of the radiation track (100 –800 microns) and declined progressively with distance with no observed effect beyond 1,100 microns [51]. However, the extent to which the bystander effect is detectable in whole blood in lymphocytes using the CBMN assay has not been conclusively demonstrated, although indirect evidence based on measurement of clastogenic factor effects following irradiation in vivo suggests this to be probable [52,53].

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Radiation biodosimetry

Biological dosimetry or Biodosimetry, is mainly performed, in addition to physical dosimetry, with the aim of individual dose assessment. It is a tool used to assess the dose received by an individual when physical dosimetry is missing or after accidental exposures due to large scale radiological events or terrorist attacks [54].The risk of developmental abnormalities and cancer risk increases with ionizing radiation (IR) [55]. In the case of accidental exposures to ionizing radiation or in the situation of a terrorist attack, it becomes essential to measure the exposure of each individual and the resulting genotoxic effects of that exposure, which depends on genetic and constitutional susceptibility. To achieve this objective, it is important to develop a reliable biological dosimeter that can be applied in a minimally-invasive manner and that can estimate dose and genetic effects accurately across the spectrum of doses that are biologically- and medically-relevant. Such a tool would be useful in classifying subjects who require urgent medical attention; i.e., those whose long-term risks of cancer might have been increased and those who need to be reassured that their exposure is unlikely to pose a serious risk in the short or long-term [56].

At present, the dicentric chromosome assay (DCA) is the only recognized tool that provides sensitive and robust biological dose estimates and has been used extensively for accidental overexposures for many years [57-60]. In case of a radiation accident, the first information comes especially from physical dose reconstruction, blood count data and from the clinical symptoms that exposed persons might display. All these sources of information may be combined with the results of biological dose assessments to obtain a clearer evaluation of the case. Biological dosimetry using cytogenetic methods is of particular importance because it takes into account inter-individual variation in susceptibility to radiation damage. As mentioned earlier, for many years, the dicentric assay performed in PBL was the only method available; and still today, it is the gold standard for cytogenetic radiation dosimetry. However, in the past years, a number of additional assays [61] have been worked out and validated, including the now well-established micronucleus (MN), translocation and premature chromosome condensation assays. More recently, molecular biomarker methods such as the gamma-H2AX assay have been proposed [62]. Numerous studies, performed both on animals and humans, have demonstrated a close relationship between dicentric chromosomes induced in peripheral blood lymphocytes (PBLs) under in vitro and in vivo conditions. This allows dose estimation of an accidentally exposed person by comparing the observed aberration yield of dicentrics to an in vitro calibration curve. The power of dicentrics for dose estimation is related to the low and constant spontaneous dicentric rate in the healthy population (about one dicentric per 1000 metaphases), and by the fact that dicentrics are specifically induced by ionizing radiation. After whole-body exposure with low linear energy transfer (LET) radiation, doses down to _0.1 Gy can be detected. However, in cases of exposure to low doses of radiation, a disadvantage of the dicentric assay is the time needed for microscopic scoring analysis of a sufficient number of metaphases. As MN can arise from exposure to various clastogenic agents in the form of acentric chromosome fragments, as well as to aneugenic agents as whole chromosomes, they are not radiation specific. As a consequence, the CBMN assay is often used as a general toxicology test [6]. However, because ionising radiation is a strong clastogenic agent, and thus a potent inducer of MN, the CBMN assay has proven to be a very reliable, thoroughly validated and standardized technique in the field of radiation biology to evaluate in vivo radiation exposure of occupational, medical and accidentally exposed individuals and to assess individual in vitro radiosensitivity or cancer susceptibility [9,11, 12, 14,60,62-73]. Radiation induced chromosome aberrations such as MN observed in PBL are mainly the result of unrepaired or misrepaired double strand breaks by the non-homologous end joining (NHEJ) repair pathway [70,74]. Biological dosimetry, based on the analysis of micronuclei (MN) in the cytokinesis-block

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micronucleus (CBMN) assay can be used as an alternative method for scoring dicentric chromosomes in the field of radiation protection. The cytokinesis block micronucleus cytome (CBMN Cyt) assay in peripheral blood lymphocytes is one of the few available techniques with the required characteristics of sensitivity, specificity, transportability, and reproducibility to be an effective biological dosimeter of ionizing radiation exposure and of genetic susceptibility to the harmful effects of ionizing radiation [72,73,75].

The CBMN Cyt assay has also been applied successfully to identify radioprotective agents and to determine the impact of nutritional status on susceptibility to the genome-damaging effects of ionizing radiation (IR) [8,10-12, 72,73, 76- 79]. Many studies have shown that the number of radiation -induced MN is strongly correlated with dose and quality of radiation. Although the DCA is very specific to radiation damage and has a low, stable background, it is limited by the extensive time and expertise required to perform the scoring. Traditionally, 500–1000 metaphase spreads are analyzed for each sample to provide sensitive (detection limit of 0.15–0.20 Gy) and accurate biological dose estimates in situations where only a small number of individuals are involved. However, this assay is extremely labor intensive and time consuming, requiring several days for analysis of a single sample. In the case of a large-scale radiological event, where potentially thousands of people may be concerned about radiation exposure, biological dosimetry using the conventional DCA is not feasible. Lloyd *et al.* [80] have suggested that the conventional DCA may be modified in such a situation, by decreasing the number of metaphase spreads analyzed, yet maintaining sensitivity to detect clinically relevant doses. In a mass casualty event, it is generally agreed that only those individuals receiving a whole-body equivalent dose of ~1.5 Gy would require any medical intervention [81]. In order to achieve that level of sensitivity, scoring only 50 metaphase spreads (or 30 dicentrics) would be required [80]. However, using such an approach would still only allow a modest increase in daily throughput using highly trained biological dosimetrists. The CBMN assay has become, in the last years, a thoroughly validated and standardized technique proposed high-throughput alternative to the DCA evaluate in vivo radiation exposure of occupational, medical and accidentally exposed individuals. Compared to the gold standard, the dicentric assay, the CBMN assay has the important advantage of allowing economical, easy and quick analysis [62]. Similar to the DCA, the CBMN assay measures damage to the chromosomes (e.g. clastogenic damage); however, a slightly different culturing procedure and scoring technique is applied. A key advantage of the CBMN Cyt assay over metaphase analysis is the ease with which micronuclei can be scored and the high percentage (up to 60%) of scoreable binucleated cells. This makes it feasible to score thousands of cells visually with unequivocal identification of MN. These characteristics are necessary to have sufficient statistical power to detect small effects [56].

The main disadvantage of the CBMN assay is related to the variable micronucleus (MN) background frequency in lymphocytes among unexposed subjects due to a variety of factors including: age, gender, smoking and diet [82] by which only in vivo exposures in excess of 0.2–0.3 Gy X-rays can be detected. Also in the large-scale Fenech study [83], investigating variables influencing baseline MN frequencies, an increase of 0.31 MN/1000 BN cells/year was measured in a male population. In females, the spontaneous MN yields are higher compared to males and the increase of MN with age is more prominent: an increase of 0.58 MN/1000 BN cells/year was observed by the Ghent group [60], this in good agreement with the increase of 0.52 MN/1000 BN cells/year reported by Fenech [83]. Investigation of the MN content using a pan-centromeric fluorescence in situ hybridization (FISH) probe showed that the age increase of baseline MN frequencies can be attributed almost completely to centromere-positive MN, reflecting an increased chromosome loss with age [60]. By using an X-chromosome-specific centromeric probe, it was shown that the X-chromosome is almost always involved in this spontaneously occurring chromosome loss [84]. This finding may explain also the gender difference observed in spontaneous MN frequencies. However, at

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clinically relevant doses of ionizing radiation (~1.5 Gy), the CBMN assay may be sufficiently sensitive to discriminate between exposed individuals and those with background levels of MN. In fact, this assay has proved to be effective in estimating unknown doses in several accidental overexposures with results in close agreement with the DCA [57, 59, 63]. In the last years, several improvements have been achieved, with the ultimate goals ; of further increasing the sensitivity of the CBMN assay for low-dose detection by combining the assay with a fluorescence in situ hybridization centromere staining technique; of increasing the specificity of the test for radiation by scoring nucleoplasmic bridges in binucleated cells and ; of making the assay optimally suitable for rapid automated analysis of a large number of samples in case of a large-scale radiation accident. The development of a combined automated MN-centromere scoring procedure remains a challenge for the future, as it will allow systematic biomonitoring of radiation workers exposed to low-dose radiation [62]. Scoring of NPBs in cytokinesis-blocked BN cells acts as a biomarker of dicentric chromosome formation. In the cytokinesis-block micronucleus cytome (CBMN cyt) assay [18], it is possible to score NPBs joining the two nuclei in a BN cell. NPBs originate from dicentric chromosomes, which are induced by misrepair of chromosome breaks. Scoring NPBs in the CBMN cyt assay for radiation biodosimetry is important because the NPB index has a lower background frequency than MN frequency; unlike MN background yields, the NPB frequency is not affected by gender; NPBs in BN cells provide a direct method of measuring asymmetrical chromosome rearrangement in cells after a single-cell division and NPBs can be scored efficiently because the CBMN cyt assay allows a large proportion of BN cells to be accumulated and furthermore, MN/NPB ratio may provide a fingerprint of specific genotoxic exposure. NPBs in lymphocytes are increased in a dose-related manner following exposure to ionizing radiation and correlate well with dicentric and ring chromosome frequencies in metaphases of the same lymphocyte cultures [62].

The CBMN Cyt assay has been adopted internationally by the International Atomic Energy Agency for IR biodosimetry for in vitro genetic toxicology testing in the pharmaceutical industry, for environmental genetic toxicology in molecular epidemiology studies, and increasingly for studying the genotoxic effects of suboptimal diet [75, 85-89]. Micronucleus induction following IR exposure in humans has been reported for cell types and tissues other than lymphocytes such as erythrocytes, hair root cells, and buccal cells [90]; however, these methods, unlike the CBMN Cyt assay in lymphocytes, have not yet been adequately validated for IR biodosimetry and will therefore not be discussed in this review. It has been shown in several studies that up to 1 Gy of low-LET (linear energy transfer) IR exposure gives an almost 1:1 ratio for expression of acentric fragments in metaphases and micronuclei in binucleated cells; however, at higher doses the efficiency of conversion of acentric fragments to MN tends to diminish possibly due to the inclusion of more than one acentric fragment within a MN or mitotic slippage of cells with multiple aberrations, which would lead to failure of nuclear division and formation of a 4N mononuclear cell that would not be scored in the conventional CBMN assay. While the dose-response for acentric fragments between 0–4 Gy tends to be linear-quadratic, that for MN tends to be closer to linear at low dose exposure (~2 Gy) [91].

Results from in vivo exposure studies on cancer patients undergoing radiotherapy showed that the CBMN Cyt assay efficiently detects the accumulated dose-related increase in chromosome damage, and the observed effects were linearly correlated with the equivalent body dose [56]. To assess the suitability of the CBMN assay for biological dosimetry, MN yields have been analyzed in PBLs of different groups of patients treated with fractionated partial body radiotherapy, for e.g. cervical cancer, prostate cancer or Hodgkin's disease. The doses estimated by MN analysis agree quite well with averaged whole body doses calculated from the radiation treatment plans [92, 93]. Studies performed in thyroid cancer patients undergoing radioiodine treatment further demonstrated that

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the CBMN assay is sensitive enough to detect low average whole-body doses from internal exposure scenarios [69,94]. As the above-mentioned patient studies have shown that the CBMN assay is a reliable biomarker for radiation exposure, the CBMN assay has been used frequently to measure radiation exposure in radiation accidents and has been applied for large scale biomonitoring [62]. In a small subset of patients was shown that MN frequency tended to return to base-line values (~30%) after 24 month of radiotherapy cessation if the induced MN frequency was less than 200 % but remained high (~170%) if the RT-induced MN frequency was greater than 200% [95]. Similar results were obtained in subsequent studies [92, 96-98].

In specific radiation accident studies, the CBMN assay was applied to assess protracted exposure, due to the incorporation of long-lived radionuclides by residents in the vicinity of the Chernobyl nuclear power plant [96] and of the Semipalatinsk nuclear test site [66]. MN frequencies measured in a large number of residents were significantly associated with the estimated internal absorbed dose. MN frequency in BN cells of people living in the vicinity of the Chernobyl reactor was associated significantly with the level of ^{137}Cs in the body and positively correlated with internal absorbed dose [96]. In the case of the Istanbul accident, dose estimates (0.7–2.7 Gy) from MN frequency in BN cells of metal scrap workers exposed to a ^{60}Co source was in excellent agreement with doses obtained from dicentrics. The results from the Taiwan incident showed that the cytogenetic effect of chronic low dose (17 cGy cumulative dose), low dose-rate gamma irradiation, could be detected using the CBMN assay in individuals living in buildings constructed with ^{60}Co-contaminated steel rods relative to controls in uncontaminated . In case of small accidents involving only few patients, the dicentric assay has generally been used to assess radiation damage soon after the accident, and only a limited number of studies on MN-based dosimetry are available. In the study describing the Istanbul accident where 10 scrap metal workers were irradiated by an unshielded former radiotherapy ^{60}Co source [97] and in the study reporting the accident of a hospital worker exposed to a 50-kV contact radiotherapy X-ray device during maintenance [63], several cytogenetic end points were scored. In both radiation accidents, blood was sampled at different time points, varying between 1 month [97] and 6 months [63], after the accident took place. In both studies, MN-derived dose estimates were in striking agreement with dose values obtained from dicentric studies. Large-scale biomonitoring studies on radiation workers, like nuclear power plant workers and hospital staff, showed that the CBMN assay, and especially the CBMN assay combined with FISH staining for centromeres can detect radiation-induced chromosomal damage at the population level for accumulated doses received occupationally exceeding 50 mSv [60,62, 64].

One of the limitations of conventional metaphase analysis and measurement of MN in BN cells is the plateau in the dose-response beyond exposure doses of 10 Gy. However studies showed that other biomarkers within the CBMN Cyt assay could be used to obtain dose estimates of exposure ~10Gy. The biomarkers within the CBMN Cyt assay that proved to be useful for this purpose included the percentage of BN cells which declined exponentially with dose, the micronuclei/ micronucleated BN cell ratio which increased linearly with dose, and the trinucleated cell/tetranucleated cell ratio which increased exponentially with dose between 0–15 Gy. In the CBMN Cyt assay, apart from MN and NPB, it is also possible to measure nuclear buds, the nuclear division index (calculated from the ratio of mono-, bi- and multi-nucleated cells), as well as the proportion of cells dying due to necrosis and apoptosis; however, the usefulness of these additional biomarkers for IR biodosimetry has not yet been adequately investigated [56].

Advantages of CBMN cytome assay in biological dosimetry:

As discussed before, one of the disadvantages of the CBMN Cyt assay is the relatively high background MN frequency (1–40%) depending on age and gender, which could limit the correct estimation of radiation exposure dose if the base-line MN frequency of an individual was not

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known beforehand [17]. The background MN frequency is caused to a large extent by MN originating from whole-chromosome loss events which could be distinguished from MN originating from acentric chromosome fragments using either pancentromere probes or anti-kinetochore antibodies, because the former would be centromere or kinetochore positive while the latter would be centromere negative [75]. IR-induced MN would be centromere- or kinetochore-negative if they originated from acentric fragments, which has been verified in several studies [98, 99]. A comparative study by Thierens *et al*. (100) showed that the IR exposure detection limit in the absence of prior knowledge of MN frequency in lymphocytes for low-LET IR for the CBMN Cyt assay without centromere detection was 0.5 Gy, but this could be lowered to 0.2 Gy if centromere detection was used to score only those MN that were centromere negative. In comparison, results from this same study estimated a detection limit of 0.5 Gy for conventional dicentric analysis and 1.0 Gy for translocation analysis using tricolor fluorescence in situ hybridization (FISH).

Another important aspect of the CBMN Cyt assay is the possibility and importance of also scoring NPBs because: the NPB index has a lower background frequency than MN frequency; unlike MN, NPB frequency is not affected by gender; NPB in BN cells provide a direct method of measuring asymmetrical chromosome rearrangement in once-divided cells; NPB in thousands of BN cells scored can be scored efficiently because the CBMN Cyt assay allows a large proportion of BN cells to be accumulated; and, furthermore, MN/NPB ratio may provide a fingerprint of specific genotoxic exposure (e.g., the presence of BN cells with MN only and the absence of cells with both MN and NPB would suggest that a high level of MN is likely due to factors other than IR exposure). It has been shown that NPBs correlate very well with dicentric and ring chromosome frequency in metaphases of the same lymphocytes [91]. Within the CBMN Cyt assay it is also possible to score MN in non-divided mononucleated lymphocytes, which represent MN originating during in vivo divisions of lymphocytes in lymph nodes and bone marrow. Previous studies have shown that this biomarker is informative under conditions of chronic IR exposure after sufficient time has elapsed for the presence of such cells to appear in the blood. The suitability of this approach was demonstrated in a study of children living the radionuclide-contaminated regions of Belarus years after the Chernobyl accident [101].

It is, however, important to note that scoring MN in non-divided mononucleated cells in the CBMN Cyt assay, unlike scoring of MN and NPB in BN cells, is not suitable for biodosimetry of acute IR exposure in the first few days following exposure. Furthermore, the time course of appearance of mononucleated lymphocytes with MN in the blood following acute IR exposure is as yet not well defined.

As it has been shown that most of the radiation-induced MN originate primarily from acentric fragments while spontaneous MN contain especially whole chromosomes [102], the application of the CBMN centromere assay, which uses a pancentromeric probe to discriminate between centromere-positive and -negative MN, substantially increases the sensitivity of the CBMN assay in the low-dose range [62] .

In the two studies performed by the Ghent group [62], it was demonstrated that the majority of spontaneous MN were centromere positive (73 and 71%, respectively) while most radiation -induced MN were centromere negative. The number of centromere-positive MN only showed a very small increase with dose (5.3 and 3.7 MN/Gy/1000 BN cells, respectively). By manual scoring of centromere-negative MN in 2000 BN cells, a detection limit, at the 95% confidence limit, of 0.1 Gy was achieved. According to the results of Pala *et al*. [102], this detection limit could be even lowered to 0.05 Gy.

Automation of the CBMN cyt assay:

A key advantage of the CBMN cytome assay is the relative morphological simplicity of the cytological and nuclear features that are scored, which makes it amenable to automated image cytometry. Compared to the labour-intensive dicentric assay, the easy and rapid scoring of MN makes this method very attractive for population triage in case of large-scale radiation accidents,

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as well as for large-scale assessment of genetic damage in radiation workers receiving a high radiation dose. Algorithms for automated MN image analysis were already developed in the 1990s [62]. Two systems that are available commercially (IMSTAR, Paris, France; Metasystems, GmbH Altlussheim, Germany) have been validated against visual scoring for MN frequency using either fluorescence or transmitted light microscopy [103,86]; however, there are no systems available yet that can measure NPBs. These systems automatically identifies BN cells by the occurrence of two adjacent similarly 4-6-diamidino-2 phenylindole -stained nuclei. In a second step, MN is scored automatically in a circular region defined around the two nuclei of the BN cell [103,104]. A further evaluation of the detected yield of MN by a manual scorer is not needed. As the recognition of a BN cell is based on the occurrence of two adjacent but unattached nuclei, this MN software module does not allow to detect NPBs. Laser scanning cytometry of CBMN assay slides using the Compucyte system (Compucyte Inc., Boston, MA, USA) has also been reported [105], but further validation is required prior to recommendation. A collaborative study performed by the Ghent group using the Metasystems software [106] demonstrates the suitability and advantage of automated MN scoring for population triage in case of large-scale radiation accidents, where it is important to distinguish and isolate severely exposed individuals (~1 Gy),who require early medical follow-up and treatment.The quality of the data obtained is very much dependent on slide preparation, and optimization of such protocols is a clear fundamental step in the success of these automated applications and the agreement between data generated within and between laboratories. Ideally, a fully-automated system using only a drop of blood from a finger stick collection should be an important goal in research of the application of the lymphocyte CBMN assay in biodosimetry, as this would mean that both minimal invasiveness and automation could be achieved [56].

Limitations of the CBMN assay as a radiobiodosimetric measurement tool:

Despite the great extent of research performed in validating the CBMN Cyt assay for IR biodosimetry, there are still some important knowledge and technological gaps, some of which are also applicable to other established biodosimeters as follows:

1. The major limitations of the CBMN assay are related to retrospective dosimetry and accidents involving partial body irradiation. The tendency to underestimate radiation doses in situations of delayed blood sampling is due to the fact that MN and dicentrics represent unstable chromosome aberrations, which have a limited in vivo persistence, especially after high doses. Therefore, these diagnostic systems are less suitable for old or long-term exposures (retrospective biodosimetry), whereas FISH analysis for stable translocations remains at present the method of choice [62].
2. A second limitation of the CBMN assay, which applies also for the dicentric assay, is related to the fact that in practice most accidents involve partial body exposures, whereby undamaged PBL present outside the irradiation field will dilute the aberration yield, leading to an underestimation of the dose of the exposed tissue. In these cases, the dicentric assay, however, allows an estimation of the unexposed part of the body by comparing the distribution of aberrations among the cells with respect to the Poisson distribution. This calculation is only useful when a significant part of the body has received little or no dose, when the exposed body parts received a relatively high dose (0.3 Gy) and when the exposure has taken place over a very short period of time. As the distribution of MN is slightly over dispersed [58], the power of an MN frequency distribution analysis with respect to a partial body irradiation is questionable and still needs further investigation.
3. One of the limitations of the CBMN Cyt assay is the requirement of a 72 h culture period to complete the assay using the standard protocol. It is theoretically possible to shorten the

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protocol to 48–56 h by adding cytochalasin-B at 24 h rather than at 48 h because a sufficient number of lymphocytes would have completed nuclear division by 48–56 h. Research is therefore required to validate a shortened 48 –56 h protocol against the standard 72 h method and to explore ways in which the mitogenic response can be accelerated [56].

4. *In vitro* IR exposure studies with lymphocytes have shown that there is considerable inter-individual variation in MN induction and other measures of chromosome damage such as metaphase analysis of dicentric chromosomes and acentric chromosome fragments. A better understanding of the degree of expected variation and the genetic and dietary factors causing such variation would be helpful to plan the statistically correct number of subjects required for construction of population-representative dose response curves and to take account of genetic and dietary variables when constructing such dose response curves [56].
5. Dose-response curves for the various types of possible IR exposures that are likely to occur in a radiation accident or occupational setting need to be constructed and compared within the same laboratories for both the MN and NPB index in BN cells. In this manner the most appropriate dose-response curve can then be used for dose reconstruction [56].

The association of CBMN Cyt assay with DNA damage and genome instability:

DNA damage is a fundamental cause of developmental and degenerative diseases. The in vitro cytokinesis-block micronucleus cytome (CBMN-Cyt) assay in human lymphocytes is one of the most commonly used methods for measuring DNA damage rates in human populations because it is relatively easier to score MN than chromosome aberrations. This technique is originally developed to study the acute effects of single environmental genotoxicants, creative applications and adaptations to the basic protocol have allowed its use in evaluating the impacts of dietary micronutrients and micronutrient combinations (nutriomes) on DNA damage [10, 72,73, 77, 107,108].

Studies show that by CBMN assay in combination with chromosome specific centromeric probes, the cells can also be analyzed for non-disjunction and aneuploidy of specific chromosomes that might be important in cancer (e.g. chromosomes 7 and 17) or in Alzheimer's disease (e.g. chromosome 21). For example, this approach significantly higher propensity in their lymphocytes to malsegregate chromosome 21, leading to elevated frequencies of chromosome 21 triploidy. This supports the hypothesis that perhaps mosaicism of chromosome 21 aneuploidy could lead to accelerated ageing of selected portions of specific tissues within the body, such as the brain. The CBMN assay has also been successfully used to show that individuals, who develop breast cancer, and their relatives, exhibit elevated sensitivity to the DNA-damaging effects of ionizing radiation [14]. This sensitivity was indicative of a defect in double-strand break repair and was observed in 10 of 11 cases of BRCA1 mutation carriers [109]. These data suggested that the assay was useful not only as a biomarker of spontaneous damage to DNA but also as a measure of DNA repair phenotype. Given the large number of different BRCA1 mutations and the 120+DNA repair genes [110]. Because MN and NPBs originate from mis-repair of double strand breaks in DNA, it is possible to use the CBMN Cyt assay to determine inter-individual variation in susceptibility to the genome damaging effects of IR. These differences in susceptibility may occur through a number of mechanisms, including defective genes involved in the error-free homologous recombinational repair pathways (e.g., BRCA1) or cell-cycle checkpoint genes (e.g., p53) as well as deficiency in endogenous or diet-derived metabolites that could quench the formation of reactive oxygen species in the cytoplasm or nucleus (e.g., caffeic acid, catechin) [109, 50,77]. Studies show a highly significant inter-individual variation in the frequency of radiation-induced BN cells with MN in otherwise healthy women. Other studies have shown that IR induced MN in BN lymphocytes are significantly elevated in women who are carriers of BRCA1 and BRCA2 mutations as well as in those women who develop non-familial breast cancer, indicating

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the utility of the CBMN assay to measure susceptibility to IR-induced chromosome damage and as a biomarker of breast cancer risk [103, 109]. Lee *et al.* [110] have also shown that MN frequency in prostate cancer patients prior to radiotherapy can vary significantly following IR challenge of lymphocytes *in vitro* and that such an *in vitro* test of IR sensitivity phenotype is predictive of gastrointestinal and genito-urinary morbidity following radiotherapy of prostate cancer. Inter-individual variation in biological response to radiation is a potential confounder for measuring physical radiation exposure dose in radiation exposure accidents if the reference laboratory's dose-response curves used for dose estimation were generated using blood samples from subjects who are not representative of the general population susceptibility. The sensitivity of the CBMN Cyt assay to inter-individual variation in susceptibility is an important characteristic of the assay because it allows individualized risk assessment to be made that takes into consideration not only physical exposure dose but also genetic and dietary factors that might alter susceptibility. For the purpose of risk assessment, it is ultimately the extent of radiation-induced DNA damage that matters, and risk of adverse health outcomes should be measured on the basis of induced DNA damage or in this case induced micronucleus frequency in binucleated lymphocytes [111].

Expression of MN could also be used as a surrogate marker of DNA hypomethylation. For example, the DNA methylation inhibitor 5-azacytidine induces under condensation of the heterochromatin regions of chromosomes 1, 9 and 16, and the specific loss of these chromosomes as MN in human lymphocytes *in vitro* [112]. The immunodeficiency, centromeric region instability, facial anomalies syndrome, which is caused by mutation in the DNA methyl transferase DNMT3B gene, is characterized by despiralization of heterochromatin of chromosomes 1, 9 and 16 and loss of this chromatin into NBUDs and MN . These events are likely to be relevant to the aging process because Suzuki *et al.* [113] demonstrated that in vitro aging of normal human fibroblasts results in concomitant demethylation of satellite 2 and satellite 3 DNA (which is abundant in the juxta-centromeric DNA of chromosomes 1, 9 and 16) and the increasing frequency of MN that specifically contained these sequences. It has been conclusively shown that in vivo aging leads to an increased MN frequency in lymphocytes and that loss of the X chromosome in females and males and loss of the Y chromosome in males are among the primary mechanisms explaining this increase [113,114]. Although it is evident that MN expression could be increased as a result of hypomethylation of satellite DNA, it is also possible that increased genome damage may be caused by hypermethylation of CpG islands within or adjacent to the promoter regions of housekeeping genes involved in cell-cycle check points and DNA repair. For example, CpG island hypermethylation of mitotic spindle check point genes such as APC, BUBR1 and hCDC4 could reduce their expression and therefore increase the possibility of chromosome malsegregation leading to MN formation [115, 116]. Silencing or loss of function of ATM, FANC and BRCA1 or BRCA2 genes involved in DNA repair could also result in higher baseline MN frequencies in human [18].

Other applications of CBMN Cyt assay

The association of CBMN Cyt assay with cancer

It is well known that cancer results from the accumulated genetic events that have the potential to be detected cytogenetically [117]. Chromosome aberrations are a result of errors in DNA repair in response to exposure to the clastogenic agent. Because cancer can take from several years to decades to develop it is not always practical to perform prospective epidemiological studies over such long periods. Therefore, there is justifiable interest in determining whether biomarkers of DNA damage might be predictive of cancer risk. The biomarkers of exposure and effect and clinical disease can all be largely influenced by susceptibility factors, which include polymorphisms that alter the activity of relevant DNA repair, carcinogen metabolism and apoptotic pathway genes, as well as dietary factors that alter the activity of these genes. Ideally, measurements are conducted in the same tissue.

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The validation of a single biomarker of DNA damage for cancer prediction requires a long period of study often involving the effort of numerous laboratories. The process of validation has been reviewed ; however, this would involve three key stages: (a) the development of standardized protocols that take into account sample acquisition, sample storage, expression time, (b) determining the most important methodological, demographic, life-style and genetic variables that influence the index measured, and finally (c) the test in case-control and prospective studies of the sensitivity and specificity of the biomarker to predicting risk of cancer generally and of a specific cancer. The only cytogenetic biomarker that has been through most of the stages outlined previously is the technique of classical metaphase analysis for measurement of chromosome aberrations (i.e. chromosome breaks and rearrangements) in human lymphocytes [118]. It is generally acknowledged that a crucial event in the initiation and evolution of cancer is the acquisition of a genomic instability phenotype. Cancer is a complex disease in which cells with altered gene expression grow abnormally, invade other tissues and disrupt their normal function. Several critical mutations in unique genes that accelerate cell division and inhibit cell death alter the balance towards the survival of the cancer phenotype and these events are now well documented for specific cancers [119]. However, the number of critical mutations accumulated within cancer cells cannot be explained solely by the normal point. It has been proposed that mutations leading to hypermutability are central to the initiation of the cancer process [119-121]. Furthermore, although cancers are apparently clonal, there could be great genetic variability between cells within a cancer clone because genetic instability occurs at the early stage of cancer [122]. A consensus is emerging that a crucial early event in carcinogenesis is the induction of the genomic instability phenotype, which enables an initiated cell to evolve into a cancer cell by achieving a greater proliferative capacity and the genetic plasticity to overcome host immunological resistance, localized toxic environments and suboptimal micronutrient supply.

the best studied mechanisms of genomic instability in cancer caused by mutation in DNA mismatch repair genes (e.g. MSH2, MLH1) that leads to the accumulation of point mutations in the DNA sequence, which are readily observed in microsatellites .Tumors exhibiting microsatellite instability are classified as MIN tumors [123]; these tumors differ from other tumors have an apparently normal karyotype . The most of the tumors, however, exhibit abnormal karyotypes involving either chromosomal rearrangement and/or aneuploidy. Those tumors that exhibit abnormal chromosome number are classified as CIN (chromosomal instability) tumors [123, 124]. Abnormal chromosome number can initially occur by a variety of mechanisms, which include: abnormal centriole number leading to multipolar mitoses, chromosome loss at anaphase as a result of kinetochore defects, malsegregation of chromosomes at anaphase as a result of defects in the separation of chromatids, mitotic slippage caused by inhibition of mitosis leading to the formation of tetraploid cells, and failure of cytokinesis following nuclear replication as a result of defects in microfilament assembly [23]. It has been shown that different carcinogens can induce either MIN or CIN genomic instability in vitro, which supports the hypothesis that specific carcinogen exposure or DNA repair defects determines the type of genetic instability in the cancers they induce [124].

An important limitation of DNA damage biomarkers in human studies is the relevance of the accessible tissue in which DNA damage is measured (e.g. erythrocytes, lymphocytes, exfoliated epithelial cells) to the cancer studied (e.g. breast, prostate, colon). Lymphocytes are excellent markers of exposure because they circulate for years or even decades through different organs and accumulate DNA damage during their lifespan [125]. Much evidence has been collected about the association between micronuclei (MN) induction and the development of cancer [126].

Results from a case–control study of lung cancer in smokers showed that both spontaneous and nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone (NNK)-induced MN are associated with increased lung cancer risk and the association

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with NPB and NBUDs was even greater [127]. These initial results indicate the potential predictive value of the CBMN Cyt assay with respect to cancer risk and validate its use as a test for detecting nutritional, environmental and genetic factors that are potentially carcinogenic. Another study confirmed that the CBMN assay was an accurate predictor of lung cancer and supported the premise that heavy smoking may have an effect on DNA repair capacity and in turn modulate the risk of lung cancer [128].

During 2002, a prospective cohort study was performed in Europe in the EU Cancer Biomarkers program, in which the predictivity with respect to cancer risk of MN frequency in lymphocytes measured using the CBMN assay was determined [23]. Also the risk for cancer in the HUMN project cohorts was predicted by MN frequency in peripheral blood. The ultimate objective of the HUMN project studies was to test the hypothesis that an elevated MN frequency in human tissues was predictive of cancer risk [129]. The results from this study provided evidence that MN frequency in peripheral blood lymphocytes was a predictive biomarker of cancer risk within a population of healthy subjects.

The association of CBMN Cyt assay with variety of Disease:

As discussed before, the CBMN assay is a very important cytogenetic test because of its feasibility and ability to detect clastogenic and aneugenic effects. The MN are DNA-containing particles that arise during mitosis and result from unrepaired DNA double-strand breaks, leading to chromatin fragments or whole chromosomes incorrectly distributed during mitosis. Several studies have reported increased MN frequencies, indicating genomic instability, in variety of disease, following paragraphs mention to some examples:

Renal Failure Patients:

Patients with CKD have signs of extensive DNA damage and an elevated risk for developing cancer [130, 131]. This risk may be related to impairment of DNA repair. DNA lesions may induce mutations in oncogenes and tumor-suppressor genes that may lead to malignancies if mutagenicity is not mitigated by repair mechanisms. Elevated frequencies of micronuclei (MN) in cultured peripheral blood lymphocytes (PBL) of patients before and after renal therapy have been found [131]. In summary, these results indicate that CKD patients show increased background levels of DNA damage, which indicate reduced DNA integrity in patients with CKD [132]. These changes were more marked in male, diabetic and non-dialyzed stage 4 CKD patients, while CKD stage 5 patients undergoing HD treatment appeared to have fewer alterations compared with those treated by PD [133]. Studies in Chronic Renal Failure (CRF) Patients showed that these patients show genomic instability as measured by increased radiosensitivity to the induction of genetic damage. The background levels of genetic damage and the net genetic damage after in vitro irradiation with 0.5 Gy were analyzed using the micronucleus assay in peripheral blood lymphocytes of 174 CRF patients and 53 controls. Results indicated that CRF patients show increased radiosensitivity and that the degree of radiosensitivity was associated with the progression of the pathological stage of the disease [134]. Among biomarkers used to detect genetic damage, the frequency of micronuclei (MN) in peripheral blood lymphocytes is considered one sensitive indicator of exposure to exogenous or endogenous sources of genetic damage. These results confirmed that CRF patients exhibit higher levels of genetic damage than normal individuals. This had been estimated by using the frequency of MN as a biomarker of effects. This biomarker has great biological relevance since MNs represent fixed genetic damage resulting from both aneugenic and clastogenic mechanisms [75] and it was considered a good surrogate marker of cancer risk [129,135-140]. Because of the monoclonal origin of growing cells in the atherosclerotic plaques [141], genomic instability would not only explain the high incidence of cancer in these patients, but it would also explain the high incidence of cardiovascular pathologies, since this illness has been associated to genomic instability [142]. This finding suggested that MNs measured in

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lymphocytes challenged with in vitro irradiation, could be considered as a biomarker of prognosis to different pathologies, including CRF.

Cardiovascular autonomic neuropathy:

In another study, the CBMN frequency of 46 patients suffering from autonomic neuropathy was compared with that of 25 healthy age and sex matched controls. The CBMN frequency in patients was found to be altered with life style and risk factors like smoking, alcoholism, diabetes mellitus, hypertension, dyslipidemia, abdominal obesity, physical activity, diet, socioeconomic status and area of residence. A strong correlation between CBMN frequencies and cardio autonomic neuropathy was observed in this study [143].

Polycystic ovary syndrome (PCOS):

Over the past decade, emerging evidence has shown that an increased chromosomal damage, as determined by CBMN assay, is correlated to the pathogenesis of metabolic and CVD. An increased micronuclei (MN) frequency has been demonstrated in peripheral blood lymphocytes of patients with polycystic ovary syndrome, a common condition in reproductive-aged women associated with impaired glucose tolerance, T2D mellitus and the metabolic syndrome (Met). However, there is yet very little information available on the association between MN levels, obesity and MetS. Several papers have demonstrated an association between increased MN frequency and polycystic ovary syndrome (PCOS) [144, 145]. PCOS is a complex endocrine condition affecting 4–8% of women of reproductive age. It is noteworthy that elevated levels of genomic instability (greater number MN and chromosome malsegregation) present in women with PCOS was positively correlated with the BMI and insulin resistance levels [144,146].

Diabetes

Studies on diabetic cases showed that As regards MN assay, no significant difference was found in MN frequency in type-1 diabetic patients as compared with controls [44]. On the contrary, significantly high levels of MN frequency were found in patients with type 2 with no microvascular or macrovascular complications [147]. It was found that in a large population of patients undergoing coronary angiography, T2D was the major independent determinant of an increased MN frequency in circulating lymphocytes of patients with ischemic heart disease [148]. It also was shown that diabetes was a significant determinant of MN levels, even when the patients were stratified according to the presence of CAD. Recently, Zu´n˜iga-Gonza´lez *et al.* demonstrated that either controlled or uncontrolled diabetic patients had ~2-fold higher frequency of MNs in buccal mucosa samples than healthy subjects. There was also evidence of an increased MN frequency among patients with uncontrolled type 1 diabetes as compared with patients with a good metabolic control. Furthermore, a significant reduction in MN was observed after folate supplementation for 30 days [149].

Cardiovascular disease:

At the present time, there is consistent evidence supporting the notion that oxidative stress-induced-genetic instability is a relevant contributor of atherosclerotic plaque development and its acute complications [150-152]. Indeed, DNA damage is present in human atherosclerotic plaque ranging from 'macro' damage, including deletions or additions of whole chromosomes or parts of chromosomes to 'micro' damage which includes loss of heterozygosity and microsatellite instability (mutations in DNA regions that may affect gene expression), DNA strand breaks and modifications of DNA (including oxidation) or DNA adducts [153- 155]. Specifically, the existence of chromosomal aneuploidy in multinucleated endothelial cells may be important in atherogenesis by strongly expressing low-density lipoproteins (LDL) receptors and increasing LDL uptake to the subendothelial intima [153]. Chromosomal aberrations can also occur in vascular smooth muscle cells of human atherosclerotic plaques, especially in unstable plaques [154]. During the last ten years, the presence of chromosomal damage in circulating cells of patients with CAD using the CBMN assay was demonstrated [142, 156]. Studies In this field showed an elevated frequency of MNs was

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significantly correlated with both the occurrence [142, 156] and the severity of coronary artery disease [142].

Clinical follow-up:

The cytogenetic monitoring, carried out on 1650 healthy subjects aimed to define some reference biological parameters of a 'normal population' to be used as a control for further analysis demonstrated a significantly shorter survival for CVD for subjects with a higher MN frequency as compared to subjects with a lower frequency [157]. Finally, findings indicated that MN levels are the significant predictors of future cardiovascular events in patients with known CAD. In a long-term follow-up prospective study of 178 consecutive patients, the relationship between tertiles of MN and the risk of major adverse cardiovascular events (cardiac death, myocardial infarction, stroke, congestive heart failure, unstable angina, coronary and peripheral revascularization) was assessed. After a mean follow-up of four years, it was found that the overall event-free survival was 77.5, 70.4 and 49.0% in patients with the low, medium and upper tertiles of MN, respectively. Results showed that patients in the upper tertile had a 2.2-fold increased risk of developing adverse cardiac events [158]. Overall, these studies indicated that an increased MN frequency was correlated to the pathogenesis of metabolic and CVD. The results supported the hypothesis that the CBMN assay may expand the prognostic power of established biomarkers for the detection and the progression from MetS to T2D mellitus and CVD [148]. Other studies have since been published showing that a higher MN frequency in lymphocytes measured using the CBMN assay is associated prospectively with increased pregnancy complications [159] and cardiovascular disease mortality [157, 158].

Occupational and environmental exposure and lifestyles

The CBMN assay is the most frequently used chromosomal biomarker in human lymphocytes which provides the opportunity for a harmonized approach for studying genotoxicity and cytotoxicity both *in vitro* and *ex vivo*, which is important for modeling and predicting *in vivo* effects in humans [18].This provides the opportunity to test, using the same system, the extent to which the in vitro test is predictive of *in vivo* genotoxic events. The adoption of the CBMN Cyt assay by the pharmaceutical industry [88, 127], its application in human biomonitoring of genotoxic exposures [129, 160, 161] and its increasing application in preventive medicine and nutrition [8, 10-13, 72,73,89 ,110, 127] and the increased investment in the automation of the CBMN Cyt assay are indicative of the increasing importance of this test [162].

It is now feasible and plausible to consider the concept of recommended dietary allowances (RDAs) using genomic stability as the main parameter on which to set these intake levels [163]. The importance of this approach is supported by the fact that: several micronutrients, such as zinc, magnesium, folic acid and vitamin B12, are required as co-factors in DNA metabolism, deficiency in these co-factors could induce important chromosomal mutations that increase cancer risk, and genomic stability is crucial for normal function of cells. We can envisage a future when the ability to use DNA damage biomarkers for predicting cancer risk will be considerably improved because of a better understanding of how dietary factors modulate genomic stability.

In a series of studies on folic acid deficiency in long-term primary human lymphocyte cultures, the interrelationship between MN, NPBs and NBUDs was carefully quantified to validate the use of these biomarkers and to determine more comprehensively the impact of folic acid deficiency, within the physiological range, on various aspects of genomic stability [27, 28, 164]. Folic acid concentration correlated significantly and negatively with all these markers of chromosome damage, which were minimized at 60–120 nM folic acid. The strong cross-correlation between MN, NPB and NBUD frequency suggests a common mechanism initiated by folic acid deficiency-induced DNA breaks, the most plausible being chromosomal instability generated by breakage– fusion–bridge cycles [23].

In a research, genetic polymorphisms of

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XRCC1, HOGG1 and MGMT and micronucleus occurrence in Chinese vinyl chloride-exposed workers was studied. Results confirmed that VCM exposure was associated with the risk of chromosomal damage. Moreover, high-age workers had more evidence of chromosomal damage determined by the MN assay. It was also observed that workers who possessed the XRCC1 194 Arg/Trp, XRCC1 280 Arg/His and His/His genotype and hOGG1 326 Ser/Cys genotype faced a significantly higher risk of chromosomal damage. These experimental evidences highlighted the usefulness of the MN assay, as a biological marker for assessing genetic damage in populations exposed to VCM, and suggest that XRCC1 and hOGG1 polymorphisms might contribute to increase the genetic damage, possibly due to reduced DNA repair function [111].

Diet is an important variable in DNA damage biomarkers, studies in vegetarian and non-vegetarian populations, who had wide differences in plasma folate, vitamin B12, vitamin C and vitamin E concentrations, indicated quite clearly that folate and vitamin B12 were more important determinants of micronucleus (MN) frequency than vitamin C or vitamin E [165]. Subsequent studies in men aged (50–70 yrs) and young adults (aged 18–20 yrs) confirmed these early observations. However, in addition, It was also showed that MN frequency was not only significantly and negatively correlated with serum vitamin B12 but also significantly and positively correlated with plasma homocysteine, a risk factor for cardiovascular disease [166].These results illustrate that dietary deficiency of folate and vitamin B12 is an important determinant of DNA damage rate and that above RDA intakes of these vitamins might be required in large subsets of a population to minimize DNA damage rate [23].

Taking smoking studies confirmed that smokers generally do not experience an overall increase in MN frequency. However, when the interaction with occupational exposure was taken into account, heavy smokers were the only group showing a significant increase in genotoxic damage as measured by the CBMN assay in lymphocytes [167]. Based on these results, some general recommendations for the design of biomonitoring studies involving smokers can be formulated. Quantitative data about smoking habit should always be collected because, in the absence of such data, the simple comparison of smokers versus non-smokers could be misleading. The subgroup of heavy smokers (~30 cigarettes/day) should be specifically evaluated whenever it is large enough to satisfy statistical requirement [167].

The presence of an interaction between smoking habit and occupational exposure to genotoxic agents should always be tested. It was shown that spontaneous and cigarette smoke nitrosamine-induced frequencies of MNi, NPBs and nuclear buds in lymphocytes of smokers who develop lung cancer are higher than those of matched smoker controls who do not develop lung cancer [127,168]. These results suggest that DNA damage biomarkers in the CBMN assay are only likely to be substantially elevated as a result of smoking in those with abnormally high susceptibility to carcinogenic effects of genotoxicants contained in cigarette smoke. Whether the elevated MN frequency in smokers with lung cancer is only due to increased susceptibility to cigarette smoke genotoxicants or also due to the presence of cancer itself will not be known until comparisons are also made for MN frequency between nonsmoker and smoker lung cancer cases [167].

As mentioned before the standard protocol for quantification of chromosomal aberrations by CBMN assay, involves culturing freshly isolated peripheral blood lymphocytes (PBL) for 72 h in complete medium prior to blocking cells during cytokinesis with cytochalasin-B (CytoB) and harvesting onto slides. This method was originally developed to study the acute effects of single environmental genotoxicants with exposures ranging from 1 to 24 h and cultures not exceeding 72 or 96 h prior to harvest of cells. Such an approach, however, is not efficacious for evaluating the impacts of micronutrients and micronutrient combinations (nutriomes) as the genomic impacts of micronutrients take at least 7 days to become evident and effects may vary with increasing time and/or genotype. Accordingly, several studies have been

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conducted whereby adaptations to the standard protocol have provided a means for assessing the impact of micronutrients, in isolation [169].

The HUMN and HUMNxL Projects:

The International Human Micronucleus (HUMN) Project (www.humn.org) was founded in 1997 to coordinate worldwide research efforts aimed at using micronucleus (MN) assays to study DNA damage in human populations [19]. The central aims were to collect databases on baseline MN frequencies and associated methodological, demographic, genetic and exposure variables, determine those variables that affect MN frequency, establish standardized protocols for performing assays so that data comparisons can be made more reliably across laboratories and countries and evaluate the association of MN frequency with disease outcomes both cross-sectionally and prospectively. In the first 10 years of the HUMN project, all of these objectives were achieved successfully for the MN assay using the cytokinesis-block micronucleus (CBMN) assay in human peripheral blood lymphocytes and the findings were published in a series of papers that are among the most highly cited in the field [167]. The CBMN protocol and scoring criteria are now standardized; the effect of age, gender and smoking status have been defined, and it was shown prospectively using a database of almost 7000 subjects that an increased MN frequency in lymphocytes predicts cancer risk. In 2007, the HUMN coordinating group decided to launch an equivalent project focused on the human MN assay in buccal epithelial cells because it provides a complementary method for measuring MN in a tissue that is easily accessible and does not require tissue culture. This new international project is now known as the human MN assay in exfoliated cells (HUMNxL). At present, a database for >5000 subjects worldwide has been established for the HUMNxL project. The inter-laboratory slide-scoring exercise for the HUMNxL project is at an advanced stage of planning and the analyses of data for methodological, demographic, genetic, lifestyle and exposure variables are at a final stage of completion. Future activities will be aimed at defining the genetic variables that affect MN frequencies; validation of the various automated scoring systems based on image analysis, flow cytometry and laser scanning cytometry; standardization of protocols for scoring micronuclei (MN) in cells from other tissues, e.g. erythrocyte and nasal cells and; prospective association studies with pregnancy complications, developmental defects, childhood cancers, cardiovascular disease and neurodegenerative diseases. Furthermore, it brought to attention the need to achieve similar advances with the MN assay in buccal cells in which there is an increasing interest because of their ease of collection, their proximity to food and air-borne genotoxicants and relevance to carcinogenesis in epithelial tissues [167]. Although this assay has been used since the 1980s to demonstrate cytogenetic effects of environmental and occupational exposures, lifestyle factors, dietary deficiencies and different diseases, important knowledge gaps remain about the characteristics of MN and other nuclear abnormalities, the basic biology explaining the appearance of various cell types in buccal mucosa samples and effects of diverse staining procedures and scoring criteria in laboratories around the world and their relationship to disease states and outcomes [170]. To address these uncertainties, the HUMN project coordinating group initiated a new international validation project for the buccal cell MN assay similar to the project previously performed using human lymphocytes (as described above) [75, 171, 172]. To distinguish it from the lymphocyte project, this project was given the acronym HUMNxL, i.e. human MN assay in exfoliated buccal cells.

Recommendations for future research:

Important issues on which future research on the MN cytogenetic assay should focus include the following.The development of a combined automated MN-centromere scoring procedure remains a challenge for the future, as it will allow systematic biomonitoring of radiation workers exposed to low doses. Furthermore, this implementation will allow, in the case of mass radiation causalities, a more accurate assessment of the exposure in a second phase- after

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early triage- when the time constraint will be less strict. It will combine high-speed MN analysis with a more accurate assessment in the low-dose range. The establishment of an international network including several cytogenetic reference laboratories establishing and optimizing International Standardization organization (ISO) standards for the conventional and automated CBMN assay seems necessary. By the creation of such a network of trained laboratories using similar equipment for MN automation and the same classifiers, standardized fixation protocols, etc., comparable results can be obtained and the throughput of automated MN scoring can be increased to allow a rapid response to large-scale radiation accidents. A European program has been started whereby multi-disciplinary biodosimetry tools, including the CBMN assay, will be developed in 15 European groups, to manage high-scale radiological casualties and to increase European capabilities in radiological incident response. Further refinement of the CBMN assay is needed to optimize its use in retrospective biodosimetry and for the analysis of cases of protracted exposure and partial body exposure. To date, only limited and diverse data are available concerning the disappearance of MN, and further research and validation is needed. Appropriate calibration curves need also to be established for more complex exposure scenarios [62]. MN assays in other tissues such as erythrocytes, nasal epithelium and hair root cells have been shown previously to be of relevance to human studies in specific scenarios (e.g. erythrocytes in micronutrient deficiency and nasal epithelium in air pollution) [173]. Protocols for these systems have been reported but these have not been standardized or validated with respect to variables that may be expected to affect baseline frequencies and in terms of prospective association with disease outcomes.

Up to now, only the lymphocyte MN assay has been tested for its sensitivity to the impact of diet and lifestyle factors and the studies reported to date are sparse. A greater effort with large well-defined populations is required to replicate these initial studies and identify those diet and lifestyle patterns that associate with reduced or increased MN frequencies. Although several studies have reported on the impact on MN frequency of common single-nucleotide polymorphisms (SNPs) in candidate genes involved in metabolic pathways that may directly or indirectly affect genome stability, most of these studies were underpowered [174]. Furthermore, it is important that genome-wide association studies using random SNP analysis be performed with large cohorts to identify unknown genes that may strongly affect MN frequency or to verify those that have been identified as being determinants of this biomarker when mutated. An important development in MN assays is the adoption of the cytome approach that not only scores MN but also captures other nuclear abnormalities such as nuclear buds and NPBs as well as capturing frequencies of necrotic and apoptotic cells as well as the proportion of cells undergoing cell division [75]. In the cytome approach, even MN in nondivided cells may need to be considered due to alternative mechanisms of MN formation such as nuclear budding or in the case of lymphocytes due to pre-existing MN expressed in aberrant *in vivo* nuclear divisions [18, 87]. The comprehensive micronucleus cytome approach is increasingly being adopted as it enables all major nuclear anomalies and cytotoxicity events to be captured simultaneously. It will take considerable effort to validate the other biomarkers in the cytome system but this will become necessary if the addition of these indices improves association with genotoxicant exposures and/or disease outcomes as has been suggested by a recent study on the association between the CBMN cytome assay biomarkers in lymphocytes and lung cancer [127,168].

During the past decade, great advances have occurred that have enabled automated scoring of MN as well as high content analysis of MN and nuclei using molecular probes that provide additional information on DNA damage mechanism (e.g. centromere and telomere detection in MN and nuclei). Procedures and reagents for these methods require standardization particularly if they are to be used on a routine basis for MN analyses.

Larger and/or longer studies are required to

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verify the results of previous studies concerning the association of MN frequency with pregnancy complications, cancer and cardiovascular disease [129, 157-159]. Prospective studies of MN in cells from the umbilical cord (at birth) with respect to association with cancers in childhood and later in life are as yet uninvestigated and deserve attention given that the carcinogenic risk from DNA damage early in life may be of great significance. Associations with other diseases such as Alzheimer's disease, Parkinson's disease and diabetes have been reported in cross-sectional studies [175, 176] but prospective data are required for ultimate validation of the MN biomarker as a predictor of these degenerative diseases.

Future studies should also explore the relationship of MN expression with changes in DNA methylation and the associated transcriptome, metabolome and proteome profiles to unravel the underlying molecular mechanisms that correlate with this DNA damage biomarker. This 'omic' data could provide valuable information on the likely origin of MN when the exposure profile is unknown or difficult to ascertain [167].

CONCLUSION

In conclusion, the activities of the HUMN and HUMNxL projects have transformed the approach to MN assay validation, standardized the performance of the assay and scoring procedures and facilitated the wide-spread adoption of this valuable technology as a tool for investigating the most fundamental pathology of the human condition, i.e. damage to the human genome. The ultimate goal is to see the validated MN assays becoming a routine diagnostic in the new disease prevention paradigms and strategies required for this new millennium based on personalized prevention of DNA damage [167]. The CBMN Cyt assay has been also demonstrated in numerous studies to be one of the best available methods for IR biodosimetry. The suitability of this method will be considerably further enhanced once the technological and knowledge gaps identified above are adequately addressed. New approaches such as centromere staining, NPB scoring and automation of MN scoring, which have been recently optimised, and are still under development will render the CBMN assay more sensitive and specific for radiation dose estimations and make it of special interest for large-scale screening applications [62].

Conflicts of interest: *none to declare.*

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