The buccal micronucleus cytome assay: New horizons for its implementation in human studies

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THE BUCCAL MICRONUCLEUS CYTOME ASSAY – NEW HORIZONS FOR ITS IMPLEMENTATION IN HUMAN STUDIES

Report of HUMN project workshop at Malaga 2023 EEMGS conference

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Abstract

In this report we provide a summary of the presentations and discussion of the latest knowledge regarding the buccal micronucleus (MN) cytome assay. This information was presented at the HUMN workshop held in Malaga, Spain, in connection with the 2023 European, Environmental Mutagenesis and Genomics conference. The presentations covered the most salient topics relevant to the buccal MN cytome assay including (i) the biology of the buccal mucosa, (ii) its application in human studies relating to DNA damage caused by environmental exposure to genotoxins, (iii) the association of buccal MN with cancer and a wide range of reproductive, metabolic, immunological, neurodegenerative and other age-related diseases, (iv) the impact of nutrition and lifestyle on buccal MN cytome assay biomarkers; (v) its potential for application to studies of DNA damage in children and obesity, and (vi) the growing prospects of enhancing the clinical utility by automated scoring of the buccal MN cytome assay biomarkers by image recognition software developed using artificial intelligence. The most important knowledge gap is the need of prospective studies to test whether the buccal MN cytome assay biomarkers predict health and disease.

Key words: buccal micronucleus, DNA damage, occupational, clinical, nutritional, automation

Abbreviations: AI artificial intelligence**, CBMN assay** cytokinesis-block micronucleus assay, **DNN** deep neural network**, MN** micronucleus or micronuclei

1.Introduction

Micronuclei (MN) are expressed in cells that have structural chromosome aberrations and/or defects in the mitotic apparatus that leads to failed segregation of chromosome fragments and/or whole chromosomes during mitosis [1]. The lagging chromosome fragments or whole chromosomes are excluded from the two main nuclei at anaphase/telophase and are ultimately surrounded by membrane to form MN. Measurement of MN in human cells has become one of the most widely used methods to measure chromosome instability and the DNA damaging effects of environmental and endogenous genotoxins [2,3]. The best validated of these methods in humans is the lymphocyte cytokinesis-block micronucleus cytome (CBMN) assay in which MN, and other related nuclear anomalies, such as nucleoplasmic bridges and nuclear buds, are scored exclusively in cells that have completed one nuclear division *ex vivo* after mitogen stimulation which are identified as binucleated (BN) cells after blocking cytokinesis with cytochalasin-B [4].

Another method to measure MN in humans is to use buccal cells which are post-mitotic epithelial cells that can be collected in a minimally invasive manner from the inside of the mouth. In this method MN and other nuclear anomalies such as nuclear buds can be observed and scored without the need of *ex vivo* culture of cells [5]. Because of the relative ease of collecting, preparing, fixing and storing buccal cells, there is growing interest in further developing and validating this assay for *in vivo* biomonitoring studies in humans. Recent reviews have shown that the relative increase in MN frequency of buccal cells induced by exposure to genotoxins, or buccal cells from people with age-related degenerative diseases such as cancer and cardiovascular disease, is similar to that observed in cytokinesis-blocked lymphocytes [6,7].

However, there are some important knowledge and technological gaps regarding the buccal MN assay that need to be resolved. The knowledge limitations include (i) lack of prospective studies showing that an elevated MN frequency in buccal cells predicts an increased risk of developmental and degenerative diseases and (ii) lack of knowledge on whether mitotic rate in the basal layer of the buccal epithelium substantially affects MN frequency. The technological gaps that suggest challenging goals for the future include (i) lack of automated systems to score MN frequency in buccal cells which is critical given the lower incidence of MN in buccal cells relative to lymphocytes, (ii) lack of image analysis algorithms that can distinguish MN from other nuclear anomalies such as nuclear buds, or distinguish between normal cells and different types of cell death such as cells that have nuclei with condensed chromatin, karyorrhexis or pyknosis. The workshop was designed to discuss the current status of the buccal MN cytome assay and determine the most important near-term and long-term goals to further validate the assay and enable its more practical application in human studies. Presentations in the HUMN Malaga workshop were given by experts on the use of the buccal MN cytome assay including the mechanisms and biology of MN formation in buccal cells, application of this assay in occupational exposures to genotoxins, the association with disease, nutrition and lifestyle and the potential challenges and opportunities for automation. Summaries of these presentations and the main points of discussion that emerged are provided below.

2. The biology of buccal cells and the buccal micronucleus (MN) cytome assay

Claudia Bolognesi reported that the buccal mucosa forms the primary barrier for the inhalation or ingestion routes and is, therefore, a high-risk site for exposure to genotoxic agents entering the body via the aerodigestive tract. The oral epithelium, which is composed of multiple layers of cells, maintains itself by continuous cell renewal whereby new cells produced in the basal layer by mitosis migrate to the surface replacing those that are shed. Basal cells impacted by genotoxic agents express the genetic damage as chromosome breakage or loss, resulting in formation of MN during nuclear division. Daughter cells with or without MN differentiate into squamous epithelial cells, then exfoliate into the buccal cavity and can be easily collected and analysed. The MN assay applied in exfoliated cells represents a minimally invasive approach to evaluate genomic damage in biomonitoring studies [5,8].

The MN assay in buccal cells was established in 1982 to evaluate the genotoxic effects induced by chewing betel quid [9]. This method has been largely applied in the last 40 years in biomonitoring human populations exposed by inhalation or oral ingestion of a variety of genotoxic and carcinogenic agents. The buccal MN test was also used to evaluate the effects of anti-cancer agents, and to study the impact of nutrition and lifestyle factors on genome integrity. A large number of studies appeared more recently on the application of the buccal MN assay in the follow-up of cancerous and precancerous oral lesions and as a biomarker of chromosomal instability in patients with cancer and/or with different chronic diseases [10]. Based on the data available, the association of MN in buccal cells with some diseases appears to be as robust as MN in lymphocytes [6].

More recently the buccal MN assay evolved into the "buccal MN cytome" method which includes the additional scoring of the different cell types and nuclear anomalies providing a comprehensive evaluation of the biomarkers of DNA damage, biomarkers of cell death, biomarkers of cytokinetic defects or arrest [5,8]. Data collected in biomonitoring occupational or environmental exposure and in clinical studies suggest an added value for the evaluation of the cytome biomarker profile as reported below.

3.The use of buccal MN cytome assay in occupational exposure studies

Georg Wultsch informed the workshop that so far about 200 studies have been published which concern the formation of MN in buccal cells of workers exposed to a variety of potentially genotoxic occupational scenarios. The first investigation with iron-exposed workers was published already 30 years ago [11]. Most studies (n=55) concern the impact of exposure to agricultural chemicals followed by workers that are exposed to petroleum and its derivatives (n=24). A similar number of investigations was conducted with medical staff (exposed to anaesthetic gases, cytostatics and radiation) (n=22). Further studies were conducted with medical students and anatomy laboratory staff who are exposed to formaldehyde (n=14). Less frequently studied groups are miners, electroplaters, welders, painters and carpenters.

Dr Wultsch and his team conducted in the last decade studies with the latter groups and found a clear positive result in individuals who work in furniture production [12] but not in electroplaters [13] and welders [14]. Also, with workers that are exposed to chicken manure (used for energy production) negative results were obtained [15]. It is notable that in all these studies the number of nuclear anomalies which reflect acute cytotoxicity in the buccal MN cytome assay (i.e. karyolysis, karyorrhexis, condensed chromatin) was significantly higher in exposed subjects. A clear increase of MN was found in cotton weavers (in Pakistan) that are exposed to cotton dust [16]. The latest study concerned the induction of DNA damage in street markers that are exposed to silica crystals and various chemicals. A clear increase of genotoxic effect with a duration of work was detected. This is the first study which demonstrated increased genetic damage in this occupational group [17]. The currently available data indicate that MN studies with buccal cells are a cost-effective, rapid and simple approach to find out if workers are exposed to genotoxic carcinogens. This method could complement the chemical exposure measurements which are currently used to control the safety of workers.

4. Association of buccal MN cytome assay biomarkers with disease and their relevance for clinical studies

In his presentation Stefano Bonassi focused on the potential clinical application of the buccal MN assay as a test to identify those patients at higher risk of degenerative diseases such as cancer. He reported on the limited extent of clinical data concerning MN frequency in buccal cells and their comparison with MN frequency in lymphocytes in cancer and non-cancer diseases. In all diseases examined, MN in lymphocytes and exfoliated cells were higher than in controls, with the exception of prostate cancer [6]. The ratio of MN frequency in subjects with disease vs controls in lymphocytes (2.3 and 2.0 for non-cancer diseases and cancer, respectively) was significantly lower than the corresponding estimates observed in exfoliated buccal cells (3.6 and 6.1). The strongest association was found for those cases in which MN were measured in cells from the same tissue in which cancer was diagnosed (e.g., oral cancer in the case of buccal cells). He discussed how to validate and translate the application of MN assays into clinical practice and presented a possible roadmap driving this process. Critical steps are the following: (a) differentiate disease patients from unaffected individuals and identify important variables that can modify the MN biomarker in healthy and disease subjects; (b) drive the transition from the use of MN assays at group level to the individual level; and (3) run prospective cohort studies and randomised controlled trials to verify that MN assays are predictive of disease and that MN frequency modification alters disease outcomes. Pragmatic trials will also be required before inclusion in routine clinical practice, to provide the decisive evidence to support their adoption by the medical and public health community.

5. Impact of nutrition and lifestyle on MN and other nuclear anomalies in buccal cells

Siegfried Knasmueller reported that only few dietary studies have been realized in which the impact of vitamins (vitamin C and provitamin A, tocopherol, folate) on buccal MN were studied and in most of them (>90%) evidence for beneficial effects were found.

The buccal MN technique was also frequently used to study the consequences of consumption of various drugs. Consistently positive effects were observed in tobacco chewers and in heavy smokers [18-20]. Interestingly, they observed an inverse correlation between the nicotine contents of cigarettes and MN formation while a positive correlation with the tar contents was observed [21]. In a well-designed older trial evidence for a synergistic effect between alcohol consumption and smoking was reported [22]. Alcohol intake per se caused no clear effects in other investigations.

Several studies showed that betel and areca nuts chewing (with and without tobacco) and consumption of khat leaves lead to increased buccal MN frequencies [23-25]. This observation may explain the high incidence of oral cancer in areas where these chewing habits are prevalent. It is also notable that synthetic derivatives of ephedrine as well as synthetic and natural cannabinoids led to increased MN frequencies in *in vitro* experiments with cells from respiratory/oral tract [26]. On the contrary, no evidence of MN induction was seen in a study which we realized in South America (Peru) with coca leave chewers (i.e. in this case even a decrease of the MN frequencies was observed) [27].

A substantial number of studies (in total 17) concern the effects of mobile phone specific electromagnetic fields. High quality studies (n=4) yielded consistently negative results. Also, in their investigation with highly controlled exposure via headphones (Knasmueller et al., unpublished) no evidence for positive results was found.

Taken together, the available data show that MN assays reflect health risks as a consequence of exposure to certain drugs; the results of dietary studies are scarce and no firm conclusion can be drawn.

6. Automation of the buccal MN cytome assay

Originally, the buccal MN technique was a simple assay in which only MN are scored. Michael Fenech explained how It eventually evolved into a complex two-stage cytome assay in which cells are first classified into seven types (Basal, Differentiated, Binucleated, Condensed chromatin, Karyorrhexis, Pyknotic, Karyolytic cells) and secondly MN and nuclear buds (NBUD) are scored in differentiated cells only [28,29]. Both the relative frequency of the various cell types and the number of differentiated cells with MNi and/or NBUD have potential diagnostic value with regards to toxic environmental exposures, poor lifestyle, malnutrition and a wide range of diseases. However, scoring this complex profile of biomarkers is laborious and limits the possibility of doing genetic toxicology studies efficiently.

Therefore, there is a legitimate need to automate some of the best validated biomarkers of the Buccal MN cytome assay and ultimately achieve a fully automated system for this purpose.

In his presentation Michael Fenech discussed which of the buccal biomarkers may be easier to measure automatically by image analysis and presented preliminary data with DAPI stained slides using the Metafer system indicating the feasibility of scoring buccal MN and binucleated cells. He noted that a key remaining question is which slide preparation and staining system is most practical and suitable to optimise accuracy of visual and automated scoring of buccal cell biomarkers.

Christian Schunck reported that artificial intelligence (AI) has become a key factor for automated microscope-based image analysis. The power of Deep Neural Networks (DNN) in the evaluation of digital image content opens unimagined possibilities for automating even complex assays such as the micronucleus cytome assays. He provided the example of how MetaSystems uses this technology in its scanning software Metafer to classify objects based on criteria determined by the algorithm. These networks are trained with large amounts of pre-classified image data determined by visual scoring of cells (supervised learning). The Buccal Micronucleus Cytome Assay scores many different cell classes and DNA damage markers Consequently evaluation of a very large number of cells is required to achieve statistically significant results. Automation of the assay would therefore be highly desirable. He emphasised that the HUMN workshop provides an ideal forum to discuss, with early career scientists and experts, the different possible approaches for automation of the Buccal Micronucleus Cytome Assay using DNN.

7. Genomic instability measured using the buccal micronucleus cytome assay is predicted by obesity, oxidative DNA damage and vitamin D in children and adolescents

The minimally invasive methodology for collecting exfoliated buccal cells is one of the most appealing aspects of the Buccal MN cytome assay because it makes it practical to use with children and adolescents. Emanuela Volpi reported on her experience using this methodology in a study aimed to determine a novel approach for predicting genomic instability via the combined assessment of adiposity, systemic inflammation, DNA oxidation and vitamin D status using a cross-sectional study with 132 participants, aged 10–18, recruited from schools and paediatric obesity clinics in London. When examining relationships between variables for all participants, markers of adiposity positively correlated with acquired oxidative DNA damage ($p < 0.01$) and genomic instability ($p < 0.001$), and negatively correlated with vitamin D ($p < 0.01$). Multiple regression analyses identified obesity ($p < 0.001$), vitamin D ($p < 0.001$), and oxidative DNA damage ($p < 0.05$) as the three significant predictors of genomic instability measured using the Buccal MN cytome assay combined score of MNi, multinucleated cells, nuclear buds and nucleoplasmic bridges. Their study concluded that non-invasive biomonitoring of genomic instability using buccal cells and predictive modelling of this phenotype in young patients with obesity may contribute to their identification and prioritisation for clinical intervention measures to improve genome integrity.

8. Discussion on knowledge gaps regarding the buccal MN cytome assay and a roadmap for its translation into practice

More than 100 participated in the workshop and subsequent discussion. There was a general interest in the use of buccal MN assay and whether it can be implemented instead of the lymphocyte MN assay as a biomarker of genotoxin exposure. This question remains unanswered because of the uncertainty of whether the mechanisms that lead to MN formation in buccal cells is the same as the mechanisms that cause MN in lymphocytes and also because of potential differences in the kinetics of MN expression between these two systems. Furthermore, although the lymphocyte CBMN assay has been shown to be predictive of disease risk in four prospective studies relating to cancer risk, cardiovascular disease mortality and pregnancy complications [30-34], no studies have been reported showing that a higher level of MN frequency in buccal cells is associated prospectively with increased disease risk. It was generally acknowledged that conducting prospective studies with the buccal MN assay should be a high priority for this biomarker.

A deeper understanding of the biology of the buccal mucosa and how this varies with aging and disease is desirable to be able to correctly interpret the changes in frequency of the various cell types and biomarkers of DNA damage and cell death. Inter-laboratory slide scoring exercises have shown that there is generally good agreement between and within labs for scoring MN in buccal cells, however, the concordance of scoring other biomarkers and cell types is not as high [35-37]. It has been suggested that the cell death biomarkers (condensed chromatin and karyorrhexis cells) which are correlated but not easy to distinguish from each other, can be combined together. The other biomarkers (basal cells, nuclear buds, binucleated cells, pyknotic cells and karyolytic cells still need more stringent criteria and training to raise concordance in scoring to an acceptable level.

Furthermore, it is vital for researchers to realise the importance of using DNA-specific stains and, when possible, molecular markers such as centromere probes, to verify the genomic origin of the MN scored and the mechanisms by which they were produced (e.g. mitotic malsegregation of acentric chromosome fragments or whole chromosomes). In addition, it is essential to be aware that the kinetics of expression of MN in buccal cells may vary depending on whether the subjects examined are exposed to acute or chronic genotoxic events because in the former one may expect only transient increase in MN frequency but in the latter elevated MN frequency may be persistent.

Understanding the kinetics of MN formation in the basal layer and the time it takes for appearance of cells with MN in the surface layers of the buccal mucosa has great relevance to the optimal time to harvest buccal cells to achieve precise measurement of buccal cell MN frequency. Given the current inadequate state of knowledge on this topic it is important that future studies are designed that have a sufficient number of harvest time points to ensure that buccal MN assay captures the full extent of MN formation both in cases of acute or chronic exposure to genotoxic events.

Theoretically, MN observed in exfoliated buccal cells are only produced in the dividing cells of the basal layer of the buccal mucosa because other cells in this tissue are unable to divide and

express MN. Therefore, any ingested genotoxins could either penetrate through buccal mucosa and directly harm the basal cells or, alternatively, they are absorbed via the digestive system and reach the buccal basal cells via the bloodstream. Understanding the route by which exogenous chemical genotoxins may reach the buccal mucosa basal layer and cause DNA damage in the basal cells is an additional aspect that deserves more attention in future research.

Much of the discussion was focused on the need of a reliable automated system to score buccal mucosa cells using AI. However, the success of using automated systems to score MN and other nuclear anomalies will depend on the quality of the slide preparation and the staining method used. There was consensus that the staining method used should be DNA specific and also stain the cytoplasm of the cells so that the cell boundaries are evident. The reasons for these requirements are first to avoid false-positive MN due to unwanted staining of non-DNA structures that resemble MN such as keratohyalin granules and secondly to identify clearly and discriminate between mononucleated and binucleated cells and also to identify basal cells which are characterised by their smaller cytoplasmic area relative to fully differentiated buccal cells. The staining that is generally considered suitable for these purposes is Feulgen for nuclei and MN, and Light Green for cytoplasm. Feulgen stain has the added advantage that it can also be visualised using fluorescence microscopy without fading which is useful to verify the true positivity of MN. An alternative fluorescence staining approach is to use DAPI which is DNA specific but on its own cannot define the cytoplasmic boundaries and, therefore, a cytoplasmic fluorescent stain such as FITC or eosin will be required. Although we are in the early stage of automation of MN cytome assays promising results using lymphoblastoid cells and skin epithelial cells have been reported for the use of artificial intelligence in scoring the CBMN cytome assay biomarkers using image flow cytometry [38-40]. Whether these technologies will be successful with buccal epithelial cells collected from humans and other species is yet to be determined.

In conclusion, we find ourselves in an interesting and exciting period in which we can better appreciate the need and the possibility of validating and consolidating the use of the buccal MN cytome assay as a reliable biomarker of DNA damage and predictor of disease risk that can be readily translated into routine clinical practice.

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THE BUCCAL MICRONUCLEUS CYTOME ASSAY – NEW HORIZONS FOR ITS IMPLEMENTATION IN HUMAN STUDIES

Report of HUMN project workshop at Malaga 2023 EEMGS conference

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Abstract

In this report we provide a summary of the presentations and discussion of the latest knowledge regarding the buccal micronucleus (MN) cytome assay. This information was presented at the HUMN workshop held in Malaga, Spain, in connection with the 2023 European, Environmental Mutagenesis and Genomics conference. The presentations covered the most salient topics relevant to the buccal MN cytome assay including (i) the biology of the buccal mucosa, (ii) its application in human studies relating to DNA damage caused by environmental exposure to genotoxins, (iii) the association of buccal MN with cancer and a wide range of reproductive, metabolic, immunological, neurodegenerative and other age-related diseases, (iv) the impact of nutrition and lifestyle on buccal MN cytome assay biomarkers; (v) its potential for application to studies of DNA damage in children and obesity, and (vi) the growing prospects of enhancing the clinical utility by automated scoring of the buccal MN cytome assay biomarkers by image recognition software developed using artificial intelligence. The most important knowledge gap is the need of prospective studies to test whether the buccal MN cytome assay biomarkers predict health and disease.

Key words: buccal micronucleus, DNA damage, occupational, clinical, nutritional, automation

Abbreviations: AI artificial intelligence**, CBMN assay** cytokinesis-block micronucleus assay, **DNN** deep neural network**, MN** micronucleus or micronuclei

1.Introduction

Micronuclei (MN) are expressed in cells that have structural chromosome aberrations and/or defects in the mitotic apparatus that leads to failed segregation of chromosome fragments and/or whole chromosomes during mitosis [1]. The lagging chromosome fragments or whole chromosomes are excluded from the two main nuclei at anaphase/telophase and are ultimately surrounded by membrane to form MN. Measurement of MN in human cells has become one of the most widely used methods to measure chromosome instability and the DNA damaging effects of environmental and endogenous genotoxins [2,3]. The best validated of these methods in humans is the lymphocyte cytokinesis-block micronucleus cytome (CBMN) assay in which MN, and other related nuclear anomalies, such as nucleoplasmic bridges and nuclear buds, are scored exclusively in cells that have completed one nuclear division *ex vivo* after mitogen stimulation which are identified as binucleated (BN) cells after blocking cytokinesis with cytochalasin-B [4].

Another method to measure MN in humans is to use buccal cells which are post-mitotic epithelial cells that can be collected in a minimally invasive manner from the inside of the mouth. In this method MN and other nuclear anomalies such as nuclear buds can be observed and scored without the need of *ex vivo* culture of cells [5]. Because of the relative ease of collecting, preparing, fixing and storing buccal cells, there is growing interest in further developing and validating this assay for *in vivo* biomonitoring studies in humans. Recent reviews have shown that the relative increase in MN frequency of buccal cells induced by exposure to genotoxins, or buccal cells from people with age-related degenerative diseases such as cancer and cardiovascular disease, is similar to that observed in cytokinesis-blocked lymphocytes [6,7].

However, there are some important knowledge and technological gaps regarding the buccal MN assay that need to be resolved. The knowledge limitations include (i) lack of prospective studies showing that an elevated MN frequency in buccal cells predicts an increased risk of developmental and degenerative diseases and (ii) lack of knowledge on whether mitotic rate in the basal layer of the buccal epithelium substantially affects MN frequency. The technological gaps that suggest challenging goals for the future include (i) lack of automated systems to score MN frequency in buccal cells which is critical given the lower incidence of MN in buccal cells relative to lymphocytes, (ii) lack of image analysis algorithms that can distinguish MN from other nuclear anomalies such as nuclear buds, or distinguish between normal cells and different types of cell death such as cells that have nuclei with condensed chromatin, karyorrhexis or pyknosis. The workshop was designed to discuss the current status of the buccal MN cytome assay and determine the most important near-term and long-term goals to further validate the assay and enable its more practical application in human studies. Presentations in the HUMN Malaga workshop were given by experts on the use of the buccal MN cytome assay including the mechanisms and biology of MN formation in buccal cells, application of this assay in occupational exposures to genotoxins, the association with disease, nutrition and lifestyle and the potential challenges and opportunities for automation. Summaries of these presentations and the main points of discussion that emerged are provided below.

2. The biology of buccal cells and the buccal micronucleus (MN) cytome assay

Claudia Bolognesi reported that the buccal mucosa forms the primary barrier for the inhalation or ingestion routes and is, therefore, a high-risk site for exposure to genotoxic agents entering the body via the aerodigestive tract. The oral epithelium, which is composed of multiple layers of cells, maintains itself by continuous cell renewal whereby new cells produced in the basal layer by mitosis migrate to the surface replacing those that are shed. Basal cells impacted by genotoxic agents express the genetic damage as chromosome breakage or loss, resulting in formation of MN during nuclear division. Daughter cells with or without MN differentiate into squamous epithelial cells, then exfoliate into the buccal cavity and can be easily collected and analysed. The MN assay applied in exfoliated cells represents a minimally invasive approach to evaluate genomic damage in biomonitoring studies [5,8].

The MN assay in buccal cells was established in 1982 to evaluate the genotoxic effects induced by chewing betel quid [9]. This method has been largely applied in the last 40 years in biomonitoring human populations exposed by inhalation or oral ingestion of a variety of genotoxic and carcinogenic agents. The buccal MN test was also used to evaluate the effects of anti-cancer agents, and to study the impact of nutrition and lifestyle factors on genome integrity. A large number of studies appeared more recently on the application of the buccal MN assay in the follow-up of cancerous and precancerous oral lesions and as a biomarker of chromosomal instability in patients with cancer and/or with different chronic diseases [10]. Based on the data available, the association of MN in buccal cells with some diseases appears to be as robust as MN in lymphocytes [6].

More recently the buccal MN assay evolved into the "buccal MN cytome" method which includes the additional scoring of the different cell types and nuclear anomalies providing a comprehensive evaluation of the biomarkers of DNA damage, biomarkers of cell death, biomarkers of cytokinetic defects or arrest [5,8]. Data collected in biomonitoring occupational or environmental exposure and in clinical studies suggest an added value for the evaluation of the cytome biomarker profile as reported below.

3.The use of buccal MN cytome assay in occupational exposure studies

Georg Wultsch informed the workshop that so far about 200 studies have been published which concern the formation of MN in buccal cells of workers exposed to a variety of potentially genotoxic occupational scenarios. The first investigation with iron-exposed workers was published already 30 years ago [11]. Most studies (n=55) concern the impact of exposure to agricultural chemicals followed by workers that are exposed to petroleum and its derivatives (n=24). A similar number of investigations was conducted with medical staff (exposed to anaesthetic gases, cytostatics and radiation) (n=22). Further studies were conducted with medical students and anatomy laboratory staff who are exposed to formaldehyde (n=14). Less frequently studied groups are miners, electroplaters, welders, painters and carpenters.

Dr Wultsch and his team conducted in the last decade studies with the latter groups and found a clear positive result in individuals who work in furniture production [12] but not in electroplaters [13] and welders [14]. Also, with workers that are exposed to chicken manure (used for energy production) negative results were obtained [15]. It is notable that in all these studies the number of nuclear anomalies which reflect acute cytotoxicity in the buccal MN cytome assay (i.e. karyolysis, karyorrhexis, condensed chromatin) was significantly higher in exposed subjects. A clear increase of MN was found in cotton weavers (in Pakistan) that are exposed to cotton dust [16]. The latest study concerned the induction of DNA damage in street markers that are exposed to silica crystals and various chemicals. A clear increase of genotoxic effect with a duration of work was detected. This is the first study which demonstrated increased genetic damage in this occupational group [17]. The currently available data indicate that MN studies with buccal cells are a cost-effective, rapid and simple approach to find out if workers are exposed to genotoxic carcinogens. This method could complement the chemical exposure measurements which are currently used to control the safety of workers.

4. Association of buccal MN cytome assay biomarkers with disease and their relevance for clinical studies

In his presentation Stefano Bonassi focused on the potential clinical application of the buccal MN assay as a test to identify those patients at higher risk of degenerative diseases such as cancer. He reported on the limited extent of clinical data concerning MN frequency in buccal cells and their comparison with MN frequency in lymphocytes in cancer and non-cancer diseases. In all diseases examined, MN in lymphocytes and exfoliated cells were higher than in controls, with the exception of prostate cancer [6]. The ratio of MN frequency in subjects with disease vs controls in lymphocytes (2.3 and 2.0 for non-cancer diseases and cancer, respectively) was significantly lower than the corresponding estimates observed in exfoliated buccal cells (3.6 and 6.1). The strongest association was found for those cases in which MN were measured in cells from the same tissue in which cancer was diagnosed (e.g., oral cancer in the case of buccal cells). He discussed how to validate and translate the application of MN assays into clinical practice and presented a possible roadmap driving this process. Critical steps are the following: (a) differentiate disease patients from unaffected individuals and identify important variables that can modify the MN biomarker in healthy and disease subjects; (b) drive the transition from the use of MN assays at group level to the individual level; and (3) run prospective cohort studies and randomised controlled trials to verify that MN assays are predictive of disease and that MN frequency modification alters disease outcomes. Pragmatic trials will also be required before inclusion in routine clinical practice, to provide the decisive evidence to support their adoption by the medical and public health community.

5. Impact of nutrition and lifestyle on MN and other nuclear anomalies in buccal cells

Siegfried Knasmueller reported that only few dietary studies have been realized in which the impact of vitamins (vitamin C and provitamin A, tocopherol, folate) on buccal MN were studied and in most of them (>90%) evidence for beneficial effects were found.

The buccal MN technique was also frequently used to study the consequences of consumption of various drugs. Consistently positive effects were observed in tobacco chewers and in heavy smokers [18-20]. Interestingly, they observed an inverse correlation between the nicotine contents of cigarettes and MN formation while a positive correlation with the tar contents was observed [21]. In a well-designed older trial evidence for a synergistic effect between alcohol consumption and smoking was reported [22]. Alcohol intake per se caused no clear effects in other investigations.

Several studies showed that betel and areca nuts chewing (with and without tobacco) and consumption of khat leaves lead to increased buccal MN frequencies [23-25]. This observation may explain the high incidence of oral cancer in areas where these chewing habits are prevalent. It is also notable that synthetic derivatives of ephedrine as well as synthetic and natural cannabinoids led to increased MN frequencies in *in vitro* experiments with cells from respiratory/oral tract [26]. On the contrary, no evidence of MN induction was seen in a study which we realized in South America (Peru) with coca leave chewers (i.e. in this case even a decrease of the MN frequencies was observed) [27].

A substantial number of studies (in total 17) concern the effects of mobile phone specific electromagnetic fields. High quality studies (n=4) yielded consistently negative results. Also, in their investigation with highly controlled exposure via headphones (Knasmueller et al., unpublished) no evidence for positive results was found.

Taken together, the available data show that MN assays reflect health risks as a consequence of exposure to certain drugs; the results of dietary studies are scarce and no firm conclusion can be drawn.

6. Automation of the buccal MN cytome assay

Originally, the buccal MN technique was a simple assay in which only MN are scored. Michael Fenech explained how It eventually evolved into a complex two-stage cytome assay in which cells are first classified into seven types (Basal, Differentiated, Binucleated, Condensed chromatin, Karyorrhexis, Pyknotic, Karyolytic cells) and secondly MN and nuclear buds (NBUD) are scored in differentiated cells only [28,29]. Both the relative frequency of the various cell types and the number of differentiated cells with MNi and/or NBUD have potential diagnostic value with regards to toxic environmental exposures, poor lifestyle, malnutrition and a wide range of diseases. However, scoring this complex profile of biomarkers is laborious and limits the possibility of doing genetic toxicology studies efficiently.

Therefore, there is a legitimate need to automate some of the best validated biomarkers of the Buccal MN cytome assay and ultimately achieve a fully automated system for this purpose.

In his presentation Michael Fenech discussed which of the buccal biomarkers may be easier to measure automatically by image analysis and presented preliminary data with DAPI stained slides using the Metafer system indicating the feasibility of scoring buccal MN and binucleated cells. He noted that a key remaining question is which slide preparation and staining system is most practical and suitable to optimise accuracy of visual and automated scoring of buccal cell biomarkers.

Christian Schunck reported that artificial intelligence (AI) has become a key factor for automated microscope-based image analysis. The power of Deep Neural Networks (DNN) in the evaluation of digital image content opens unimagined possibilities for automating even complex assays such as the micronucleus cytome assays. He provided the example of how MetaSystems uses this technology in its scanning software Metafer to classify objects based on criteria determined by the algorithm. These networks are trained with large amounts of pre-classified image data determined by visual scoring of cells (supervised learning). The Buccal Micronucleus Cytome Assay scores many different cell classes and DNA damage markers Consequently evaluation of a very large number of cells is required to achieve statistically significant results. Automation of the assay would therefore be highly desirable. He emphasised that the HUMN workshop provides an ideal forum to discuss, with early career scientists and experts, the different possible approaches for automation of the Buccal Micronucleus Cytome Assay using DNN.

7. Genomic instability measured using the buccal micronucleus cytome assay is predicted by obesity, oxidative DNA damage and vitamin D in children and adolescents

The minimally invasive methodology for collecting exfoliated buccal cells is one of the most appealing aspects of the Buccal MN cytome assay because it makes it practical to use with children and adolescents. Emanuela Volpi reported on her experience using this methodology in a study aimed to determine a novel approach for predicting genomic instability via the combined assessment of adiposity, systemic inflammation, DNA oxidation and vitamin D status using a cross-sectional study with 132 participants, aged 10–18, recruited from schools and paediatric obesity clinics in London. When examining relationships between variables for all participants, markers of adiposity positively correlated with acquired oxidative DNA damage ($p < 0.01$) and genomic instability ($p < 0.001$), and negatively correlated with vitamin D ($p < 0.01$). Multiple regression analyses identified obesity ($p < 0.001$), vitamin D ($p < 0.001$), and oxidative DNA damage ($p < 0.05$) as the three significant predictors of genomic instability measured using the Buccal MN cytome assay combined score of MNi, multinucleated cells, nuclear buds and nucleoplasmic bridges. Their study concluded that non-invasive biomonitoring of genomic instability using buccal cells and predictive modelling of this phenotype in young patients with obesity may contribute to their identification and prioritisation for clinical intervention measures to improve genome integrity.

8. Discussion on knowledge gaps regarding the buccal MN cytome assay and a roadmap for its translation into practice

More than 100 participated in the workshop and subsequent discussion. There was a general interest in the use of buccal MN assay and whether it can be implemented instead of the lymphocyte MN assay as a biomarker of genotoxin exposure. This question remains unanswered because of the uncertainty of whether the mechanisms that lead to MN formation in buccal cells is the same as the mechanisms that cause MN in lymphocytes and also because of potential differences in the kinetics of MN expression between these two systems. Furthermore, although the lymphocyte CBMN assay has been shown to be predictive of disease risk in four prospective studies relating to cancer risk, cardiovascular disease mortality and pregnancy complications [30-34], no studies have been reported showing that a higher level of MN frequency in buccal cells is associated prospectively with increased disease risk. It was generally acknowledged that conducting prospective studies with the buccal MN assay should be a high priority for this biomarker.

A deeper understanding of the biology of the buccal mucosa and how this varies with aging and disease is desirable to be able to correctly interpret the changes in frequency of the various cell types and biomarkers of DNA damage and cell death. Inter-laboratory slide scoring exercises have shown that there is generally good agreement between and within labs for scoring MN in buccal cells, however, the concordance of scoring other biomarkers and cell types is not as high [35-37]. It has been suggested that the cell death biomarkers (condensed chromatin and karyorrhexis cells) which are correlated but not easy to distinguish from each other, can be combined together. The other biomarkers (basal cells, nuclear buds, binucleated cells, pyknotic cells and karyolytic cells still need more stringent criteria and training to raise concordance in scoring to an acceptable level.

Furthermore, it is vital for researchers to realise the importance of using DNA-specific stains and, when possible, molecular markers such as centromere probes, to verify the genomic origin of the MN scored and the mechanisms by which they were produced (e.g. mitotic malsegregation of acentric chromosome fragments or whole chromosomes). In addition, it is essential to be aware that the kinetics of expression of MN in buccal cells may vary depending on whether the subjects examined are exposed to acute or chronic genotoxic events because in the former one may expect only transient increase in MN frequency but in the latter elevated MN frequency may be persistent.

Understanding the kinetics of MN formation in the basal layer and the time it takes for appearance of cells with MN in the surface layers of the buccal mucosa has great relevance to the optimal time to harvest buccal cells to achieve precise measurement of buccal cell MN frequency. Given the current inadequate state of knowledge on this topic it is important that future studies are designed that have a sufficient number of harvest time points to ensure that buccal MN assay captures the full extent of MN formation both in cases of acute or chronic exposure to genotoxic events.

Theoretically, MN observed in exfoliated buccal cells are only produced in the dividing cells of the basal layer of the buccal mucosa because other cells in this tissue are unable to divide and

express MN. Therefore, any ingested genotoxins could either penetrate through buccal mucosa and directly harm the basal cells or, alternatively, they are absorbed via the digestive system and reach the buccal basal cells via the bloodstream. Understanding the route by which exogenous chemical genotoxins may reach the buccal mucosa basal layer and cause DNA damage in the basal cells is an additional aspect that deserves more attention in future research.

Much of the discussion was focused on the need of a reliable automated system to score buccal mucosa cells using AI. However, the success of using automated systems to score MN and other nuclear anomalies will depend on the quality of the slide preparation and the staining method used. There was consensus that the staining method used should be DNA specific and also stain the cytoplasm of the cells so that the cell boundaries are evident. The reasons for these requirements are first to avoid false-positive MN due to unwanted staining of non-DNA structures that resemble MN such as keratohyalin granules and secondly to identify clearly and discriminate between mononucleated and binucleated cells and also to identify basal cells which are characterised by their smaller cytoplasmic area relative to fully differentiated buccal cells. The staining that is generally considered suitable for these purposes is Feulgen for nuclei and MN, and Light Green for cytoplasm. Feulgen stain has the added advantage that it can also be visualised using fluorescence microscopy without fading which is useful to verify the true positivity of MN. An alternative fluorescence staining approach is to use DAPI which is DNA specific but on its own cannot define the cytoplasmic boundaries and, therefore, a cytoplasmic fluorescent stain such as FITC or eosin will be required. Although we are in the early stage of automation of MN cytome assays promising results using lymphoblastoid cells and skin epithelial cells have been reported for the use of artificial intelligence in scoring the CBMN cytome assay biomarkers using image flow cytometry [38-40]. Whether these technologies will be successful with buccal epithelial cells collected from humans and other species is yet to be determined.

In conclusion, we find ourselves in an interesting and exciting period in which we can better appreciate the need and the possibility of validating and consolidating the use of the buccal MN cytome assay as a reliable biomarker of DNA damage and predictor of disease risk that can be readily translated into routine clinical practice.

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