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Micronucleus Formation in Primary Oropharyngeal Epithelial Cells Reveals Mutagenicity of Cement Dusts

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Abstract. *Background/Aim: Epidemiological studies showed an increased risk of developing laryngeal head and neck squamous cell carcinoma (HNSCC) for employees working in the construction business. This suggested a causal link between exposure to cement particles and development of HNSCC but data were missing. Materials and Methods: We established an Organisation for Economic Co-operation and Development (OECD) guideline 487-conform micronucleus assay (MNA) using oropharyngeal mucosa-derived primary epithelial cells (OPCs) ex vivo. OPCs from healthy mucosa of 52 donors were cultured in vitro and incubated with serial concentrations of two common cement particles. Mitomycin C was used as a soluble positive control, and TiO_2 and DQ12 were used as negative and positive particle controls. Binucleated cells were counted and the mitotic index (MI) was determined. Subsequently, micronuclei-containing binucleated cells (MN+) were counted. Results: Cement particles, in concentrations not significantly reducing ex vivo proliferation according to mitotic index, dose-dependently increased micronuclei formation. Conclusion: Through the establishment of an OECD guideline 487 conform MNA, we demonstrate the mutagenic effects of cement on human OPCs.*

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Based on previous epidemiological studies, exposure to cement dust may be associated with upper respiratory tract (URT) disease, including airway obstruction or malignant tumour growth such as development of head and neck squamous cell carcinoma (HNSCC) and laryngeal carcinoma in particular (1, 2). Furthermore, the relative risk of cement workers for developing cancer of the larynx may increase with smoking (2). In contrast, other studies have found no association between cement dust exposure and respiratory health effects (3-6). Cement is widely used in the construction business (7) and consists of natural raw materials such as limestone (calcium carbonate), chalk (calcium oxide), clay (quartz), and other added constituents, *e.g.* granulated blast furnace slag, natural puzzolana, but also fly ash or burnt oil shale. Consequently, people working in the cement production and construction workers are exposed to cement dust. Because toxicological data on possible cement dust-induced effects on cells *in vitro* are still rare, the objective of the present study was to investigate whether cement dust induces DNA damage and mutations.

Genotoxic properties of the cements and of some of the compounds present within the various commercially available cement preparations (chromium salts, limestone and chalk) have been described earlier. Reports of cement associated genotoxicity data in the literature regarding human cells are from either leukocytes or cell lines (which are already in a malignant state). These effects were mostly judged to be either artefacts or to appear in inadequate concentrations. Moreover, it remained an open question if genotoxic events translate into mutations wherefore these data were interpreted as inconclusive. Direct evidence showing induction of mutations in human primary oral, pharyngeal or laryngeal mucosa cells is therefore needed to show the so far missing link between genotoxicity of cements and increased HNSCC frequency in workers

occupationally exposed to cement dust. In order to examine the mutagenic effects of substances on the oral cavity in general, a test system using oropharyngeal mucosa-derived primary epithelial cells (OPCs) would have been needed, but so far, no primary human epithelial cells have been tested *in vitro* to get more physiological results.

Evans *et al*. have described micronuclei as a quantitative measure of chromosome damage (8); they are defined as acentric chromosome fragments developed during cell division, without being part of the nuclei of daughter cells (9). Investigations on micronuclei frequencies support the widely accepted assumption that micronuclei are a product of early event(s) in human carcinogenic processes including DNA double-strand breaks, especially in oral regions (10-14). Furthermore, a reproducible detection of micronuclei rate in different cytological preparations has been shown (15-17). Because of its sensitivity and reliability, the micronucleus assay is suitable to detect DNA damage and to analyse the potential cytogenetic damage of environmental pollutants (18-20).

Here, we describe a micronucleus assay using OPCs to gain reproducible and valid mutagenicity testing results of two representative cement dusts *ex vivo*. Subsequently, we show that cement dusts induce DNA damage in cultured OPCs *ex vivo*.

Materials and Methods

Patients. After receiving patient's informed consent, 52 patients were included in this study. In none of the cases chemotherapeutic agents had been administered before biopsy. Specimens of the oral cavity or the oropharynx were taken during surgical treatment procedures under general anaesthesia. Specimen sampling was realized using tubes containing 10 ml cell-culture medium 1 (medium-1), a phenol red- and flavin-free RPMI1640 (Biochrom®, Berlin, Germany) supplemented with 10% calf serum (FCS, Biochrom), nystatin, penicillin, and streptomycin (Sigma-Aldrich, Munich, Germany). The samples were immediately transferred into the laboratory and were instantly processed under flavin-protecting conditions.

Materials. Keratinocyte-serum free medium (K-SFM), bovine pituitary extract, and recombinant human epithelial growth factor (EGF) were purchased from Invitrogen (Karlsruhe, Germany), and bovine fibronectin from Sigma. Mitomycin C was purchased from Baragena (Baden-Baden, Germany), cytochalasin B and 4',6-diamidino-2-phenylindole-dihydrochloride (DAPI) from Sigma.

Cement and reference dusts. Cement dusts, coded as H1 and G, were obtained from Verein Deutscher Zementindustrie e.V. (VDZ, Düsseldorf, Germany). Quartz DQ12 (Dörentruper Quartz; SiO_2) and Titanium-IV-oxide (TiO_2; Degussa, Düsseldorf, Germany) were obtained from Dr. Roel Schins of the "Institut für Umweltmedizinische Forschung" (IUF, Düsseldorf, Germany). The two cement dust samples were provided by VDZ. After size-separation to obtain only particles smaller than 10 μm, size-distribution analyses confirmed both preparations contained only particles with a median diameter between 1.0 and 2.0 μm and a sufficient number of ultrafine particles.

Cement dust G (Puzzolan) has a high content of SiO_2 (29.9%) and apart from manganese (1,144 ppm) and beryllium (2.63 ppm) a lower content in metals than cement dust H1. Cement dust H1 (Portland cement) is characterized by moderate silica content (SiO_2, 16.3%) and is rich in chrome (429 ppm), nickel (211 ppm), vanadium (192 ppm), cobalt (26 ppm), and zinc (714 ppm).

Cement and reference dust preparation and administration. Prior to the experiments, cement and reference dusts TiO_2 and DQ12 were heat-sterilized at 215˚C for 6 h in a Memmert heater (Memmert, Schwabach, Germany), suspended in medium, and sonicated for 15 min in an ultrasonic waterbath (Sonorex Bandelin, Berlin, Germany) to ensure uniform suspensions. Dilutions of the cements were prepared in medium-2 (K-SFM containing 10 ng/ml EGF) and added immediately to the OPCs samples.

Handling of OPCs. Upon arrival in the laboratory, OPCs were handled under flavin-protecting conditions (illumination only by sodium-discharge lamps with excitation wavelength of λ=589 nm). The mucosal specimens were minced and transferred into very low concentration trypsin/EDTA for enzymatic disintegration over night at 4˚C. Washed by 5-min centrifugation at 300 × *g* and the pellets were carefully re-suspended in fresh medium-1. The mucosal cells were seeded into fibronectin-coated tissue-culture dishes (TPP, Trasadingen, Switzerland), and cultured at 36.5˚C, 3.5% CO_2 in a humidified atmosphere until sufficiently growing out and reaching confluency. The initial medium-1 was step-wise replaced by medium-2 until passage 1. Then, the OPCs were grown for the next two passages (two to four weeks) in medium-2. The OPCs of passage 3 were seeded into 24-well microtiter-plates (TPP) and incubated for three days until performing the MN assay.

OECD guideline 487-conform micronucleus assay. On day 3, supernatants were aspirated and OPCs of the 52 subjects were cultured each in 1.00 ml of either medium-2 (soluble negative control; sNC), medium-2 containing mitomycin C (100 pM, positive control; sPC), different concentrations of G or H1 cement dusts, TiO_2 (100 μg/cm^2, particle negative control; pNC), and DQ12 (100 μg/cm^2, particle positive control; pPC). After 24 h, cytochalasin B was added to block cytokinesis. After further 24 h, the OPCs were fixed with ethanol and DNA was stained by adding 200 μl/well DAPI of a freshly prepared solution in phosphate buffered saline (50 μg/ml). To guaranty adequate assay conditions not affecting proliferation of OPCs we evaluated 100 OPCs in each of the wells under an inverted fluorescence microscope and determined the mitosis index (MI; the mean number of nuclei per cell). The proportion of bi-nucleated OPCs (BN) was calculated by the formula BN=100*MI–100. Two investigators independently counted the micronuclei presented in 1,000 bi-nucleated cells in each of duplicate wells.

Statistical analysis. Data were expressed as median and interquartile range (IQR). The individual counting results for micronuclei formation were transferred into Excel™ (Microsoft, Unterschleißheim, Germany) and micronuclei formation relative to control was calculated. Statistical differences were analysed using Wilcoxon signed rank *t*-test for paired samples or the nonparametric Mann-Whitney *U*-test for unpaired samples (GraphPad, Inc. V6, La Jolla, CA, USA), correlations using SPSS Statistics 20 for Windows version 20.0.1 (SPSS Inc., Chicago, IL, USA). A *p*-value below 0.05 was considered as statistically significant (*).

Table I. *Mitosis index (MI) of oropharyngeal mucosa-derived primary epithelial cells (OPCs) in cytochalasin-blocked MN assay (24 h). MI, the ratio of the number of nuclei per 100 OPCs, and the 25th and 75th percentile from 48 subjects grown ex vivo are shown.*

Substance tested	Concentration	Median	25th Percentile	75th Percentile
Medium		1.23	1.19	1.28
Mitomycin C	100 pM	1.18	1.15	1.23
Cement H1	50 μg/cm^2	1.23	1.18	1.29
	100 μg/cm^2	1.24	1.17	1.28
	200 μg/cm^2	1.23	1.19	1.29
Cement G	50 μg/cm^2	1.26	1.18	1.28
	100 μg/cm^2	1.23	1.18	1.29
	200 μg/cm^2	1.24	1.20	1.29
TiO_2	100 μg/cm^2	1.14	1.12	1.18
DQ12	100 μg/cm^2	1.17	1.13	1.21

Results

Primary oropharyngeal OPCs proliferate ex vivo. First, the mitosis index (MI) was determined as a measure of the effect of the tested substances on proliferation to have controlled test conditions not affecting normal cell function, *i.e.* proliferation. According to MI, this was the case in 48 of 52 OPCs as they showed unaffected proliferation *ex vivo* after treatment with a medium containing 100 pM mitomycin C (sPC), cement dust G and H1, TiO_2, and DQ12 compared to medium (sNC; Table I). Among these 48 OPC samples, the MI in sPC, pNC and pPC was slightly decreased compared to sNC, whereas cements G and H1 slightly increased MI compared to pNC and pPC but not compared to the medium. This demonstrates absence of toxicity and hence appropriate test conditions for cements G and H1 in the tested concentrations.

Clinical characteristics of OPC donors. Thirty-five OPCs were from male donors (72.9%), and 13 from females (27.1%; p=0.011). Thirty-five OPCs were from tumour patients (72.9%) and 13 OPCs from people without malignancy (27.1%). Ten donors were non-smokers (20.8%), and 7 had a history of ≤10 pack years (PY) tobacco smoking (14.6%), 7 of >10 to 30 PY (14.6%), and 22 of >30 PY (45.9%). 2 smokers (4.1%) provided no detailed information about their smoking habit. Regarding alcohol consumption, 7 patients drank no alcohol (14.6%), 27 drank ≤40 g/d (56.3%), and 11 >40 g/d (22.9%). However, 3 patients (6.2%) gave no information about their alcohol consumption.

Within our sample, we observed a significant difference concerning self-reported smoking habits. Male patients in general had a higher tobacco consumption than females (p=0.0002), and patients with tumour had a higher PY compared to those without malignancy (p=0.002), and smoking correlated with alcohol consumption (p=0.004).

Micronuclei assay. First, induction of MN formation in OPCs by sPC (100 pM mitomycin), was compared to medium (Figure 1). Mitomycin treatment compared to sNC increased the number of MN. Fifteen to 25% of 42 OPCs were bi-nucleated (median, 21%; p<0.001). Treatment with mitomycin C reproducibly increased the number of micronuclei in bi-nucleated cells from 1.8% (median) in control medium to about threefold (median, 5.0%; Figure 1A). According to the 2-σ criterion for the variability within all medium controls (126.9%), 130% MN were set as cut-off value for the minimal response rate required to be assessed as "significantly induced MN formation". Consequently, further 6 OPC donors were excluded as mitomycin C did not induce MN formation exceeding 130% of sNC.

In addition to the soluble control, we also tested DQ12 (Quartz, SiO_2) and TiO_2 as particle positive (pPC) and negative control (pNC), respectively (Figure 1). All tested concentrations of both cements significantly increased MN formation in the 42 OPCs compared to medium (p<0.001). The increase induced by H1 was dose-dependent, although the effect varied among the OPCs tested (Figures 1A, B). Unexpectedly, both particle controls failed regarding a desired clear-cut distinction between MN induction by pPC and lack of induction in pNC (Figure 2). TiO_2, the designated pNC, induced >130% MN formation in 13/42 OPCs (TiO_2 +) whereas only 29/42 OPCs showed – as expected – no increase compared to medium (TiO_2 –; Figure 2A, B). Contrary, the pPC DQ12 failed to induce MN formation >130% of sNC in 25/42 OPCs classified as DQ12 – (Figure 2C, D).

Nevertheless, there were no distinct differences between the subgroups TiO_2 –, TiO_2 + and DQ12 +, DQ12 – regarding their response to cement dusts tested (p>0.05). However, the TiO_2 – group showed a lower and more homogeneous reactivity to both cement dusts. OPCs not responding to TiO_2 were further subdivided into groups without distinct MN induction by DQ12 (TiO_2 –/DQ12 –; n=19) and with response to DQ12 (TiO_2 –/DQ12 +; n=10)

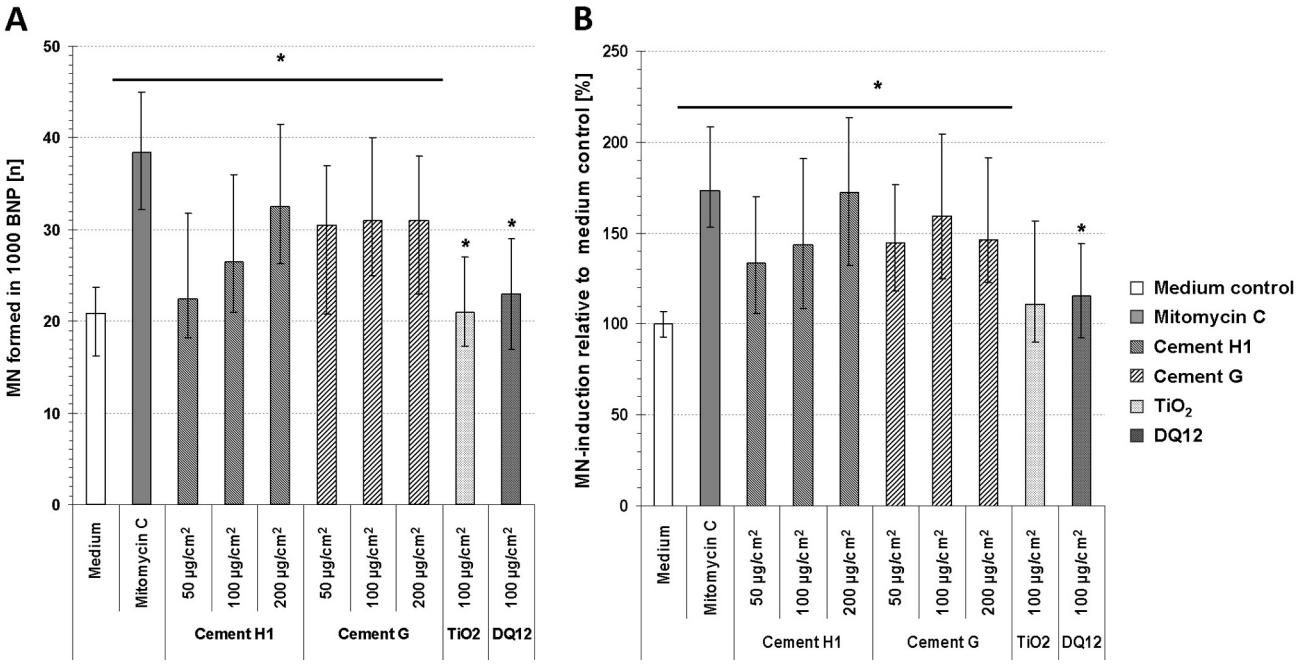

Figure 1. *Twenty-four hour incubation with increasing concentrations of cement particles significantly increased the numbers of micronuclei (MN) in oropharyngeal mucosa-derived primary epithelial cells (OPCs) detected after 24-hours of mitosis blockage with cytochalasin B. The median and interquartile range of the total number of (A) MN detected per 1000 bi-nucleated cells/well and (B) MN formation normalised to medium control of 42 OPCs showing valid MN formation according to sufficient response to mitomycin C (100 pM). A p-value below 0.05 was considered statistically significant (*).*

treatment. The same was applied for those responding to TiO_2 (TiO_2 +/DQ12 – *versus* TiO_2 +/DQ12 +; n=6 and n=7, respectively). Analyses confirmed that the OPC's response to cement dust was not affected by their strength in response to particle controls (Figure 2E, F).

Parameters potentially affecting MN formation in OPCs. To address the question which parameters might affect the OPC's response to cement, we focused on donors' clinical characteristics. We asked whether suffering from HNSCC affects the test outcome (Figure 3A, B). The 11 healthy donors showed a higher response to both cement H1 and G than HNSCC patients whereas the response intensities to cement G differed only slightly. Overall, having a tumour did not significantly affect the outcome of the MN assay (p>0.05). As only 4/10 females but 27/32 males included suffered from HNSCC (n=31/42), we looked at sex-specific differences. Comparison of OPCs from 32 males and 10 females (Figure 3C, D) revealed insignificant differences in MN formation (p>0.05). Whereas OPCs from females had slightly increased MN formation in particle controls, OPCs from males showed a slightly higher response to mitomycin C and cement G. Contrary, the response of females to cement H1 was more homogenous and significant already at lower concentrations compared to OPCs from males.

Next, we investigated the impact of smoking and alcohol consumption on MN formation. No clear difference was observed between donors with <40 g/d (n=24) and ≥40 g/d alcohol consumption (n=15; Figure 3E, F). Regarding smoking history ≤30 (n=19) or >30 pack years (n=20), there were non-ambiguous differences in the OPC's response to soluble and particulate controls, and both cement dusts. The 19 donors with <30 PY showed generally higher and significant induction of MN in response to particle controls and cement dusts independent of their concentration (Figure 3G, H; p>0.05). Moreover, they showed increased MN formation with increasing cement dust concentration (p>0.05).

Discussion

Using an Organisation for Economic Co-operation and Development (OECD) 487-conform MN assay in OPCs, a significant increase in MN induction by two representative cements was demonstrated. The dose-dependent MN induction was slightly affected by sex, alcohol consumption and tumour status and was not differed between the groups of patients defined according to their response to pNC and pPC. This result adds some evidence from *ex vivo* investigations using human OPCs, the cells who are expected

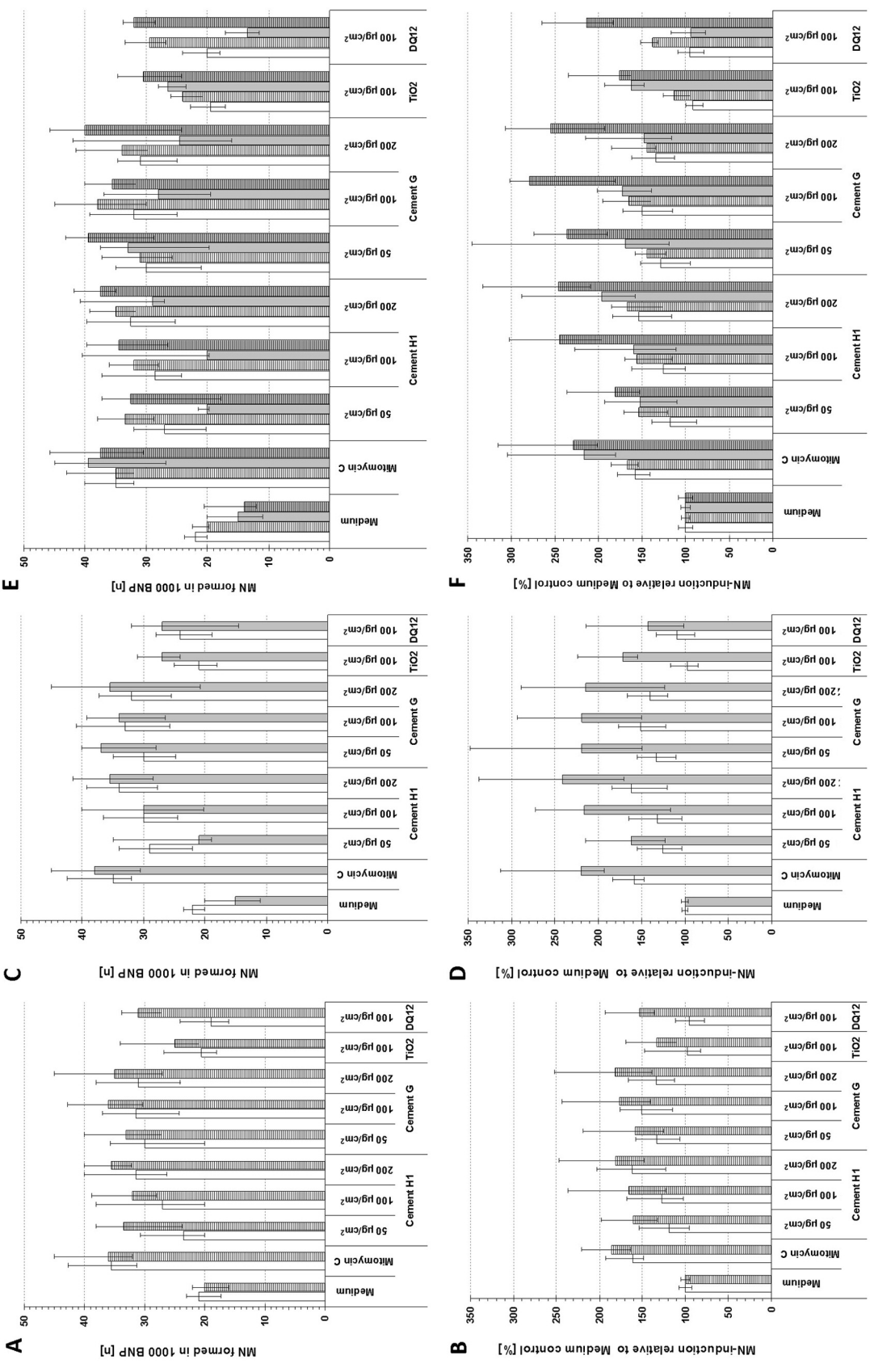

Figure 2. *Micronucleus formation in oropharyngeal mucosa-derived primary epithelial cells (OPCs) differs not in subgroups defined according to their response to particle controls TiO_2 and DQ12. Total number of MN formed in 1000 bi-nucleated cells/well and MN relative to negative control of OPCs (A, B) having either a negative (n=29) or a positive response to TiO_2 (n=13); (C, D) having either a negative (n=25) or a positive response to DQ12 (n=17); (E, F) in subgroups combining both responses† are displayed. The results are median and interquartile range. †TiO_2 –/DQ12 – defines OPCs showing <130% response to TiO_2 and DQ12 (n=19); TiO_2 –/DQ12 + no response to TiO_2 but >130% response to DQ12 (n=10); TiO_2 +/DQ12 – response >130% to TiO_2 but no response to DQ12 (n=6); TiO_2 +/DQ12 + response >130% to both TiO_2 and DQ12 (n=7).*

to be the vulnerable cells prone to carcinogenic effects of cement dust, to the often discussed link between cement dust-exposure and increased frequency of HNSCC and larynx cancer in particular (2, 4, 21).

Many previous investigations have shown cement dusts causing adverse health effects in the aerodigestive tract of building and construction workers, including granulomas, emphysemas, sinusitis, bronchiectatic lesions, and fibrosis (22-24). Induction of some of these adverse health effects has also been confirmed in animal tests (25-27). Moreover, the lung capacity and respiratory volume (ventilatory lung function) of workers in the cement industry is reduced, while the risk for developing chronic bronchitis is elevated (28-30).

However, besides the lower airways, several epidemiological studies have indicated a higher risk for developing squamous cell carcinoma of the oral cavity, the pharynx, and the larynx (31-33). This risk seems to be associated with higher exposure to insulation materials, tar, bitumen, and especially to cement dust (2, 4, 21). As the relative risk of cement workers for developing laryngeal cancer increases also with smoking, smoking may represent a confounder because of the damage caused to the ciliary epithelia and the slower clearance. Multivariate analyses, however, have provided evidence that exposure to cement dust is an independent predictor for larynx cancer (2, 4, 21). Susceptibility of subjects to genotoxic noxes and mutagens involved in HNSCC development is influenced by multiple molecular and genetic factors affecting DNA repair capacity (see 34 for review). The mutagenic effects of cements as demonstrated here points towards a causal involvement in HNSCC initiation. A prolonged exposure to high concentrations of cement dusts hence should increase the frequency of HNSCC in cement-dust exposed professions, *e.g.* construction workers, which indeed have to face a more than four-fold risk for larynx cancer (2, 4, 21).

In technical terms, our MN assay utilizing human primary OPCs provides evidence for the superiority of primary non-cancerous cells compared to (for instance, by transient transfection) immortalized cells, and in particular those cells exposed to the compound(s) of interest. So far, genotoxicity of a single compound is mostly tested *in vitro* according to the OECD guideline 487 suggesting use of cell lines (35-38). Though it is questionable, whether immortalized cell lines reflect the reactivity of epithelial cells under physiological conditions, since they are highly selected, immortalized cancer cells with a high grade of genomic instability, have increased DNA repair capacity (PARP, telomerase etc.) and other dysregulating changes causing their malignant state. Therefore, only the testing of primary human cells might allow proper toxicity and mutagenicity analysis of a given compound as other cells (already malignant cells) might not respond in a way indicating their mutagenic impact.

Despite of necessary modifications, we established a micronucleus assay using human primary OPCs meeting all OECD 487 requirements: As our MN assay appears to be potentially useful to analyse mutagenic effects exerted by other noxes on OPCs, results based on our new micronucleus assay should be more convincing than data obtained from cell lines when used for risk assessment. The OPCs showed sufficient proliferation *in vitro* and were also unaffected by 24 h exposure to known and potential mutagens. Commonly, 21% of OPCs were bi-nucleated. Furthermore, we included only OPCs showing a significantly increased MN formation due to sPC mitomcyin C (100 pM) compared to sNC for evaluation ($p<0.01$). In addition, we tested designated particle controls (100 μg/cm^2) on each OPC. DQ12, the pPC, has a genotoxic potential probably due to silica's carcinogenicity in man. The respirable crystalline silica was classified as group 1 carcinogen in 1997 (39). Its genotoxic effects are based on the ability to generate hydroxyl radicals (40-42). Titanium-IV-oxide (TiO_2) served as pNC (43). Against our expectations, TiO_2 was not inert and induced MN formation in a substantial proportion of OPCs (n=13; 31.0%). However, the major subgroup of 29 OPCs (69.0%) had MN counts within the 2-σ-range (<130% compared to sNC).

Also, in the literature, TiO_2 has been shown to have mutagenic or at least genotoxic and inflammatory properties that depended on *e.g.* particle size and protein content of the samples during preparation (44-46).

However, DQ12 failed to be a reliable inducer of MN formation in OPCs of some donors. Only a small subgroup of 17 OPCs (40.5%) showed significantly increased micronuclei formation. According to the 2-σ criterion, 25 OPCs (59.5%) had no significant MN induction by DQ12. In summary, there was a lower increase in MN formation by Quartz DQ12 mostly found to be below TiO_2 effects.

The four subgroups defined by the response to particle controls (TiO_2 –/DQ12 –; TiO_2 –/DQ12 +; TiO_2 +/DQ12 –; TiO_2 +/DQ12 +) were not substantially different regarding the effects observed following treatment of OPCs with cements.

Concerning both cement dusts, we found a significant induction in MN formation. Significantly increased numbers of MN >130% above MN formation in sNC was not uniquely observed in each donor's OPC and all concentrations, *i.e.* both cement specimens differed in this respect as cement G exerted MN induction not in every OPC. In general, cement H1 displayed a clearer dose-response relationship (lower concentrations of H1 exerted lower effects than the higher ones). At the donor-individual basis, however, the lowest concentration of cement H1 (50 μg/cm^2) sometimes failed to induce significantly increased MN formation. This might result from different composition of H1 and G and a lower mutagenicity of H1. Overall, the induction of micronuclei by cements was negative only in approximate 20% of OPCs. In other words, both cements induced MN formation above the level observed in TiO_2 (pNC) and, more surprisingly, above those of DQ12 (pPC).

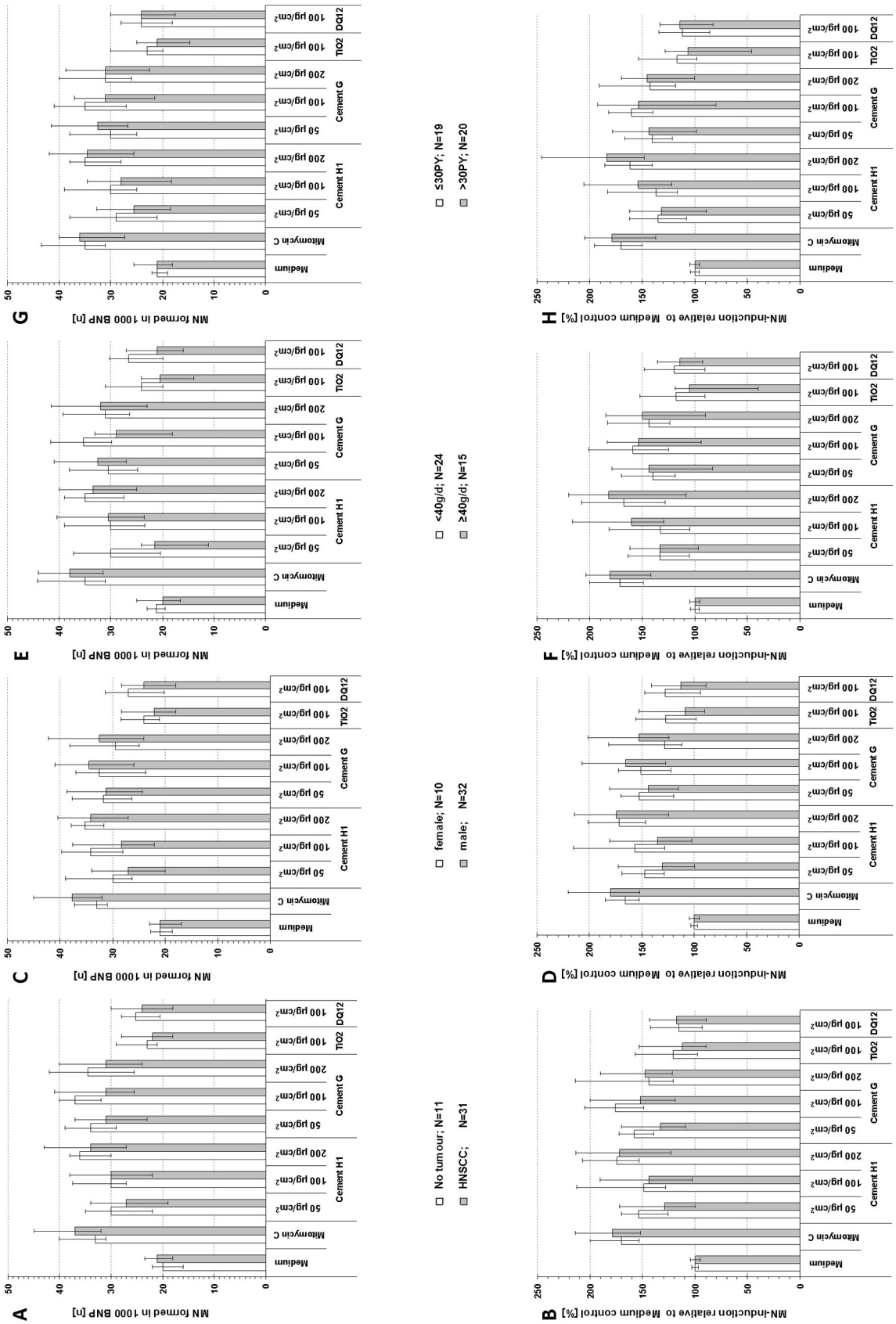

Figure 3. *Micronucleus formation in oropharyngeal mucosa-derived primary epithelial cells (OPCs) in various subgroups defined according to (A, B) malignancy status, no malignancy versus head and neck cancer; (C, D) males versus females; (E, F) alcohol consumption, ≤40 versus >40 grams per day; (G, H) smoking history of ≤30 versus >30 pack years. The results are median and interquartile range of micronuclei numbers counted in 1000 bi-nucleated cells (upper row) or relative to negative control (lower row).*

There were differences in the strength of the effects related to sex, malignancy and to a prolonged exposure to tobacco smoke (Figure 3). Especially, in the case of cement G, some OPCs had lower numbers of bi-nucleated cells and micronuclei. This was obvious in OPCs reacting to 100 μg/cm^2 of TiO_2 with significant increases in MN formation. This left-shift of the dose-response curve might reflect an increased susceptibility to mutagenic effects (higher responses regarding MN formation following treatment with lower cement concentrations) but also increased cytotoxicity causing reduced MN formation at higher concentrations of cement G but not cement H1.

Regarding risk assessment, detection of increased levels of MN formation based on our new MN assay should more convincingly indicate the ability of cement dust to induce mutations than data obtained from cell lines. As genotoxic events and mutations like MN formation are early milestones on the road to cancer development, long-term exposure to cement dust may also increase mutational load of epithelial cells lining the upper airways *in vivo*. This together with epidemiological data pointing to increased risk for development of larynx cancer (2, 4, 21) could lead to consider cement dust as carcinogenic at least in a subgroup of exposed persons.

Conclusion

Based on maintained proliferation and ability to detect dose-dependently increased MN formation, the here presented OECD 487-conform assay can be judged as a stable assay allowing reliable detection of micronuclei formation in OPCs treated by cement dust. As long conclusions are possible in the absence of a clear negative reference dust for experimental control, both cement dusts should be designated “mutagenic in primary human mucosa cells”. Adjusting the working conditions for cement workers to reduce exposure to cement dust appears to be recommended.

Conflicts of Interest

All Authors declare no potential conflicts of interest regarding this study.

Authors’ Contributions

GW designed and coordinated the study in close collaboration with MB, RG and AD and supervised experiments and counting of micronuclei. GW performed statistical analysis and wrote together with JP and MK the first draft and the final version of the manuscript. All Authors read and approved the final manuscript.

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