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State of art of micronuclei assay in exfoliative cytology as a clinical biomarker of genetic damage in oral carcinogenesis: A systematic review and meta-analysis

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common oral malignancy, often preceded by oral potentially malignant disorders (OPMDs). Currently, no clinical biomarker exists to predict malignancy, necessitating OPMD follow-up. Habits and environmental factors, such as smoking, and alcohol consumption, influence OSCC onset. Increased micronuclei (MNs) formation has been observed in the development of OSCC. Non-invasive diagnostic tests like exfoliative cytology offer painless and regular monitoring options. This study evaluates the impact of tobacco, alcohol, and pesticide exposure on MNs occurrence in exfoliative cytology-collected oral mucosal cells, assessing their potential as non-invasive biomarker for OSCC development prediction and monitoring in high-risk patients. Despite results from this meta-analysis supporting the existence of a stepwise increase from controls to patients with OPMD to OSCC, the translation of these findings into clinical practice is limited due to intra- and inter-individual heterogeneity, as well as methodological variability in MNs quantification. Various factors contribute to this heterogeneity, including demographic variables, methodological variability of different laboratories, staining techniques, sample collection location, and patient characteristics. All these points were discussed to provide further insights and improve standardization for future studies.

1. Introduction

Cancer is a disease with a great social and economic impact that is increasing worldwide. In the oral cavity, oral squamous cell carcinoma (OSCC) is the most prevalent cancer among all oral malignancies [1]. OSCC may be the first clinical manifestation or may be preceded by oral potentially malignant disorders (OPMDs). OPMDs include leukoplakia, oral submucous fibrosis, and oral lichen planus, among others. These disorders are different from each other and present unpredictable biological and clinical behavior. As a result, the risk of malignization of OPMDs varies between 0.6 and 36.4 % [2–4]. Currently, there are no clinical biomarkers available to predict malignancy, so it is mandatory to follow-up patients with OPMDs. Therefore, it is necessary to identify patients who may be at an increased risk of progression to OSCC and monitor them over time.

The oral cavity is an accessible location to explore for screening purposes. However, OSCC is often diagnosed late. This results in OSCC having a low 5-year survival rate and high morbidity [5]. Therefore, early detection of OSCC and OPMDs is critical to improving the prognosis [6].

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Previous studies have shown how the accumulation of genetic damage can lead to a process known as oral carcinogenesis. This process involves the appearance of cytological (atypia) and structural changes in tissues (dysplasia) [7]. Progression from normal mucosa to cancer is mostly attributed to damaging genetic events, mainly mutations and loss of heterozygosity [8]. Epithelium is a self-renewal tissue and each cycle may represent an opportunity for somatic mutations to occur [9]. In addition to this, oral mucosa is continuously exposed to a wide range of environmental mutagens. Indeed, oral cancer is a multifactorial disease influenced by environmental, genetic, and epigenetic factors. Its impact worldwide varies due to population habits such as smoking and alcohol consumption [10]. Tobacco contains various chemicals including genotoxic carcinogens. These substances can cause DNA mutations and promote tumor growth [5]. Moreover, alcohol consumption can damage epithelial cells and increase epithelial membrane permeability. It is well known that there is a synergistic effect between alcohol consumption and smoking, amplifying the risk of suffering OSCC [11]. In addition to these primary risk factors, there are other factors that have been associated with head and neck cancer. These include occupational exposure to polycyclic aromatic hydrocarbons, engine exhaust fumes, textile dust, and asbestos [12].

This evidence has recently been summarized in a new proposed model of oral carcinogenesis. Martins-Chaves et al. defined the "big-bang model of punctuated evolution". Evolution to OSCC is not triggered by the accumulation of random genomic alterations, but by the occurrence of true specific driver events. Among them, mutations in the TP53 gene, and in general reparative genetic defects, represent a striking point in oral carcinogenesis [5,13]. It is hypothesized that nonsense TP53 mutations may represent one of the earliest somatic alterations, followed by loss of heterozygosity on chromosomes 3p, 9p and 17p with irregular epithelial stratification, drop-shaped rete ridges and premature keratinization [13]. The oral epithelium is a self-renewing tissue that periodically renews its cells, resulting in random transient mutations. DNA repair genes that start off harboring mainly nonsense mutations are damaged by genetic susceptibility or exposure of the oral mucosa to environmental mutagens. Intralesional heterogeneity is increased when genomic maintenance is altered, leading to the accumulation of distinct clonal populations. These populations then evolve in a neutral manner until epigenetic modifications, such as abnormal methylation and altered mRNA editing, further promote the growth of abnormal clones. These aberrant clones exhibit dysregulated cell fate and survival because of an abrupt increase in pathogenic mutations and chromosomal abnormalities [9,13].

Exposure to mutagenic agents can lead to notable chromosomal aberrations. One of these aberrations is the formation of micronuclei (MNs), which are small nuclei originating from chromosomes lagging behind during anaphase or from fragments of acentric chromosomes. MNs are formed when a chromosome or fragment is not incorporated into the nuclei of daughter cells during mitosis in the basal layer of the epithelium. As a result, these MNs persist in the cytoplasm without any structural connection to the main nucleus [14]. Numerous studies have investigated the presence of MNs, as well as the number of cells with MNs in exfoliated epithelial cells of the oral mucosa, specifically in smokers and patients with OPMDs and dysplasia. These studies have found a correlation between a higher number of MNs and progression to OSCC [15–17]. Thus, the detection of MNs could be an indicator of the risk of OSCC development [18].

At present, clinical examination and biopsy remain the gold standard for the diagnosis of OPMDs and OSCC. Histology allows differentiation between benign abnormalities and dysplastic/malignant changes, which facilitates early diagnosis of cases with worse prognosis [19]. On the other hand, follow-up of oral mucosal lesions in patients with risk factors, such as smoking and alcohol, is challenging [20]. There are patients who may require multiple biopsies to be taken over time, with a consequent increase in costs and discomfort for both patients and health systems.

Recently, there has been increasing interest in non-invasive diagnostic tools. These tests are simple to perform and painless, which makes them more attractive as they allow the monitoring of the lesions [19,21]. In addition, some patients may present multiple or large lesions, also requiring multiple samplings. MNs testing have also been performed to investigate the effects of anticancer agents, nutrition, and genome integrity [22]. These techniques are also minimally invasive and have relatively low cost and provide a reliable quantitative analysis of genotoxicity affecting the oral cavity during carcinogenesis.

In light of these considerations, exfoliative cytology has emerged as a safe and effective procedure to acquire and characterize cells from the oral mucosa surface. Exfoliative cytology is the microscopic examination of cells collected by brushing the surface of oral mucosa [23].

Therefore, the aim of this systematic review and meta-analysis is to evaluate the quantitative impact of tobacco, alcohol consumption, and pesticide exposure on the occurrence of MNs in oral mucosal cells collected by exfoliative cytology. In addition, we aimed to investigate whether patients with OPMDs and OSCC present more MNs than patients without these oral lesions. This will provide valuable information about their potential as a non-invasive biomarker for predicting the development of OSCC. As well as the validity of this technique to perform non-invasive monitoring in patients with OSCC-associated risk factors.

2. Materials and methods

This systematic review and meta-analysis was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) [24]. This systematic review was registered in PROSPERO Database with the registration number CRD42022334382.

2.1. Sources of information and search strategy

Three databases were considered to identify eligible studies: PubMed, Scopus, and Web of Science. A combination of keywords was generated and adapted to each database to conduct the search (Supplementary Table 1). The search for eligible studies began on 01 June 2022 and was updated on 15 May 2023. The list of references resulting from the search strategies in the aforementioned databases was reviewed. Duplicate references were identified and removed using EndNote software (EndNote X9.3.2, Clarivate Analytics). Subsequently, a manual check for additional duplicates was performed. Furthermore, the reference lists of relevant included papers were manually screened to identify additional eligible articles.

2.2. Focused questions

This systematic review and meta-analysis aims to answer the following focused questions:

1. Is there a difference in the presence of MNs or micronucleated cells (MNC) in oral exfoliative cytology samples among individuals exposed to known risk factors such as smoking, alcohol consumption, smokeless tobacco, and pesticides, compared to those not exposed?

2. Is there a difference in the presence of MNs or MNC in oral exfoliative cytology samples of individuals diagnosed with OPMDs or OSCC compared to healthy individuals without these conditions?

The eligible studies were considered based on the Population (P), Exposure (E), Comparison (C), and Outcome (O), as described below:

(P): Population undergoing exfoliative cytology testing of oral mucosa and subsequent analysis of MNs in these samples.

(E): For the first objective, exposure to known OSCC risk factors such as smoking, alcohol consumption, smokeless tobacco, and pesticides exposure were considered. For the second objective, being diagnosed with OPMD or OSCC was taken into account.

(C): For the first aim, the comparator was considered to be those

subjects not exposed to the aforementioned risk factors. For the second goal, the comparator was healthy individuals without a diagnosis of OPMD or OSCC.

(O): Quantitative evaluation of oral cavity MNs or MNC in both the exposure (E) and comparison (C) groups.

2.3. Eligibility criteria

Articles were selected based on the following inclusion criteria: (1) studies published between the years 2000 and 2023; (2) articles published in English; (3) case-control studies; (4) studies involving patients with specific risk factors (smokers, drinkers, non-smoked tobacco users, or pesticide exposure) and/or patients diagnosed with OPMD or OSCC; and (5) studies conducted in humans.

We excluded those studies that evaluated risk factors such as environmental or occupational conditions, exposure to X-rays, systemic diseases associated with MNs formation such as coronary artery and chronic kidney disease, obesity, diabetes, autoimmune diseases, colonrectal, oesophageal, blood, skin, lung, and breast cancer [25]. Systematic reviews, case reports, case series, and in-vitro or animal studies were excluded. Studies that assessed MNs in different cell types other than the oral cavity were also excluded.

2.4. Data extraction

Two authors (FP and FS) conducted the searches independently. They reviewed the resulting list of references to determine which studies met the inclusion/exclusion criteria described above. Initially, the title and abstract of each study were assessed, and those deemed appropriate were further evaluated by reviewing the full text. To assess the agreement between the two reviewers, the calculation of k- agreement was performed at the end of this step. In cases where disagreements arose, a third author (SG) participated to provide clarification and resolve any discrepancies. This stage was also supervised by VCAC and FFVS.

Independently, two reviewers (VCAC and FFVS) performed the data extraction in Excel spreadsheets. A joint session with a third author (AILP) aimed to identify any discrepancies between the extracted data. Discrepancies were resolved during the same session after careful evaluation of the full text of the studies. The following information was obtained: name of the first author, year of publication, country where the study was conducted, mean and standard deviation (SD) of MNs and/or MNC and method of staining assessment, and the number of subjects included in both the exposed and comparator groups. In addition, from each study, the target risk factors such as tobacco and alcohol consumption, pesticide exposure or diagnosis of OPMD or OSCC, were recorded.

2.5. Risk of bias assessment

The methodological quality of the selected manuscripts was analyzed using the Newcastle-Ottawa scale (NOS) for case-control studies. The following seven items were taken into consideration: (1) Case definition, (2) representativeness of the cases, (3) selection of controls, (4) definition of controls, (5) comparability of cases and controls on the basis of the design or analysis, (6) ascertainment of exposure, and (7) same method of ascertainment for cases and controls. Studies with NOS scores 0–3, 4–6, and 7–8 were considered as high, moderate, and low risk of bias respectively [26].

2.6. Synthesis of results

A meta-analysis was performed for pooled means and SDs separately for MNs and MNC. MNs and MNC were reported differently among studies, expressed as number of observations over a total number of counted cells. To overcome this issue, meta-analysis was performed estimating standardized mean differences (SMD) and relative 95 % Confidence Intervals (95 % C.I.) by Hedges' g weighted data and effect sizes were graphically represented by forest plot in fixed or random effects models, based on heterogeneity (Cochran's Q test quantified by I^2 index, respectively $I^2 \geq 50$ % or <50 % for fixed or random effects model) [27].

Some studies included different study subgroups, for example, different types of non-smoked tobacco products *vs* the control group. In these cases, means and SDs were combined employing the formula from the Cochrane Handbook for Systematic Reviews of Interventions version 6.3 [28]. Meta-analysis was carried out separately for control *vs* smoked tobacco, electronic cigarettes (e-cigarettes), non-smoked tobacco, alcohol users, tobacco and alcohol together, pesticides exposure, OPMD, and OSCC. To furtherly assess the heterogeneity among the included studies, differences were investigated and grouped as moderators.

Sensitivity analysis was conducted for the following factors: [1] publication year, [2] country of publication, and [3] MNs or MNC staining assessment and [4] NOS risk of bias score. The statistical significance of the differences among subgroups was evaluated using the ANOVA Q-test [29]. To examine the influence of individual studies on the overall SMD, the "leaving one out" method was used. This approach involved systematically removing each study from the analysis and assessing the impact on the results [30].

In addition, a funnel plot was generated to visually assess the presence of publication bias. To further analyze the publication bias, the trim and fill analysis [31], Egger's test [32], and safe N test [33] were conducted. These methods provide statistical measures to evaluate and adjust for potential publication bias in the meta-analysis.

3. Results

3.1. Selection of studies

The last search was conducted on 15 May 2023 yielding a total of 2004 references, of which 691 remained after eliminating duplicates. Subsequently, the investigators reviewed all titles and abstracts, and 481 references were excluded because they were outside the scope of the present systematic review. A total of 210 studies were assessed for eligibility and 144 references were discarded after reading them at full text. The reasons for exclusion are listed in Supplementary Table 2. Of these, 66 references were selected for the systematic review and meta-analysis. The flow diagram is shown in Fig. 1. The obtained k-agreement value of 0.82 indicated excellent agreement between the two reviewers.

3.2. Risk of bias assessment

Of the 66 included references, 25.75 % (17 studies) were considered low risk, 65.15 % (43 studies) moderate risk, and 9.10 % (6 references) high risk.

In detail, "adequacy of case definition" was inadequate in 4 studies (5.97 %); "representativeness of the cases" was the most common inappropriate feature among included studies, accounting for 48 studies (72.72 %); "selection of controls" in 18 studies (27.27 %) was classified as inadequate; "definition of controls" was inappropriate in 27 studies (40.90 %); "comparability cases/controls" was rated with 2 points in 15 studies (22.72 %) because the confounding factors were controlled while 8 studies (12.12 %) failed in this assessment and were marked as inadequate; "ascertainment of exposure" in 7 studies (10.60 %) was classified as inappropriate; and "same method of ascertainment" was adequate in all the studies (Supplementary table 3).

3.3. Main findings and synthesis of the results

Selected studies were published between 2000 and 2022. Of the 66 studies, 34 were conducted in Asia, of which 30 were carried out in India. Brazil was the second most frequent country of origin, with 13

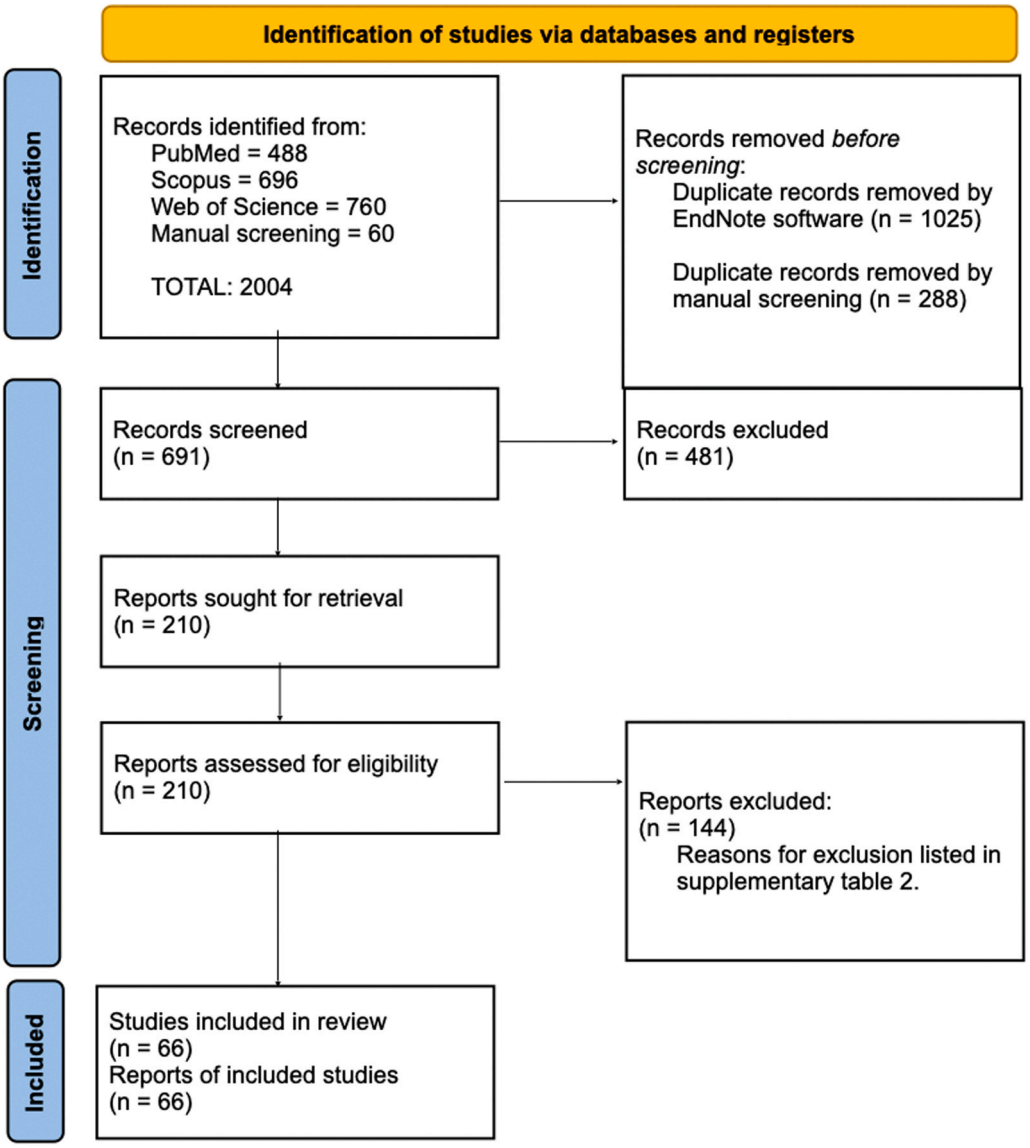

Fig. 1. PRISMA 2020 flow diagram for new systematic reviews which included searches of databases and selection process.

published studies.

Oral MNs assay was performed to investigate the genotoxic effects of smoked tobacco in 22 studies, followed by pesticides (16 studies), chewed tobacco (14 studies), and e-cigarettes (4 studies). MNs were also analyzed in patients with OPMD and OSCC (13 studies in each case). Four studies included mixed groups of OPMDs. MNs were assessed in oral submucous fibrosis (OSMF) in 4 studies with 136 cases, leukoplakia (LP) in 4 studies recruiting 116 patients, and only 2 studies included oral lichen planus (OLP) patients with a total of 92 patients. MNC were evaluated mainly in LP, comprising 186 cases, followed by OSMF (60 cases) and erythroplakia (60 cases).

Concerning the staining employed technique Giemsa was the most commonly used (22 studies) followed by Feulgen staining (15 studies). MNs and MNC were counted respectively in 46 and 32 studies since 12 studies assessed both outcomes. MNs were reported as MNs/1000 in 23 studies while MNC as MNC/1000 in 13 studies. Table 1 collects the characteristics of included studies while Table 2 summarizes the total number of participants included in each subgroup analysis.

The initial meta-analysis combined individuals who were exposed to pesticides, smoked or chewed tobacco, consumed alcohol or used e-cigarettes with individuals who served as control subjects and were not exposed to these factors. The tested population yielded overall a higher number of MNs (49 studies including a total of 1654 not exposed *vs* 1871 exposed participants - SMD=2.16, 95 %CI=1.79–2.52, p-value<0.001, random effects model I^2=95.14, Fig. 2) and MNC (39 studies including a total of 1958 not exposed *vs* 2370 exposed participants – SMD=2.21, 95 % CI=1.64–2.78, p-value<0.001, random effects model I^2=98.17, Fig. 3).

Below, we will summarize the most important results regarding the presence of MNs and MNC for each of the risk factors studied (Table 3):

- *Effect of alcohol on MNs*: Only 3 studies contributed to this outcome meta-analysis [34–36]. Patients who consumed alcohol had a significantly higher number of MNs than those who did not drink alcohol (SMD=2.34, 95 %CI=0.46–4.22, p-value=0.015, random effects model I^2=94.12).
- *Effect of alcohol on MNC*: Only 2 studies contributed to this outcome meta-analysis [37,38]. The meta-analysis showed that MNC was higher in alcohol consumers, but the results were not significant (SMD= 6.05, 95 %CI=-5.39–17.48, p-value=0.3, random effects model I^2=99.73).
- *Effect of e-cigarettes on MNs*: Four studies [39–42] analyzed this outcome. The meta-analysis showed a higher number of MNs in patients using e-cigarettes than in control patients (SMD=0.92, 95 %

Table 1
General characteristics of included studies.

Publication year	Number of studies	Country	Number of studies	Staining	Number of studies	Risk factor	Number of studies	Counting technique	Number of studies
2000	2	America	19	Feulgen	15	Alcohol	5	MN/50	1
2001	1	Asia	34	Fluorescent	11	Chewed tobacco	14	MN/100	3
2002	0	Europe	13	Giemsa	22	Electronic cigarette	4	MN/300	1
2003	1			Papanicolau	13	OPMD	13	MN/500	7
2004	1			Schiff	5	OSCC	13	MN/1000	23
2005	0					Pesticides	16	MN/1500	1
2006	2					Smoked tobacco	22	MN/2000	9
2007	1					Smoked tobacco and alcohol	7		
2008	2					Smoked and chewed tobacco	5	MNC/100	5
2009	4							MNC/300	1
2010	2							MNC/500	2
2011	3							MNC/600	1
2012	2							MNC/1000	13
2013	2							MNC/1500	2
2014	4							MNC/2000	6
2015	5							MNC/3000	2
2016	4								
2017	3								
2018	3								
2019	10								
2020	4								
2021	8								
2022	2								

Table 2
Total number of participants per each risk factor or oral disease.

Group	MNs	MNC
Control	2326	2495
Alcohol	65	162
Smoked tobacco	516	469
Smoked tobacco and alcohol	94	198
Smoked and chewed tobacco	75	374
E-cigarettes	94	60
Chewed tobacco	282	549
Pesticides	745	558
Oral potentially malignant disorders	428	378
Leukoplakia	116	186
Oral submucous fibrosis	136	60
Oral Lichen Planus	92	-
Erythroplakia	-	60
Oral squamous cell carcinoma	350	139

CI=0.41–1.43, p-value<0.001, random effects model I^2=61.26). When accounting for geographic bias, heterogeneity decreased to 0 %. The fixed effects model for Europe revealed a SMD of 1.15, 95 % CI=0.78–1.51, p-value<0.001. In this comparison, two studies implemented Giemsa and the other two performed Papanicolau (PAP) (SMD_{PAP} > SMD_{Giemsa} ANOVA Q-test p-value=0.049). The funnel plot included 0 trimmed studies and Egger's p-value=0.421 confirmed the absence of publication bias. Safe N test resulted in 29 studies failing in supporting this outcome to revert significance.

- *Effect of e-cigarette on MNC*: Three studies investigated also MNC in e-cigarette users [39,41,42]. Meta-analysis resulted in a SMD of 0.44, 95 %CI=-0.19–1.06, p-value=0.17 in random effects model, with an heterogeneity result of I^2=66.32 %. NOS score resulted inversely correlated to the effect size, meta-regression p-value=0.069. Two studies performed Giemsa and meta-analysis resulted in I^2=0 % resulting in a SMD of 0.12, 95 %CI=-0.32–0.56, p-value=0.6 in fixed effects model. Safe N test resulted in only one study to revert to non-significant results.
- *Effect of chewed tobacco on MNs*: Eight studies analyzed this outcome [35,43–49]. The meta-analysis resulted in SMD of 4.14 and 95 % CI=2.75–5.52, p-value<0.001. This meta-analysis was characterized by high heterogeneity (I^2=96.86 %). Accounting for staining, 3 studies used Giemsa and 3 PAP with relatively small changes on SMD (4.78 and 3.49 respectively). Only one study employed Feulgen (SMD=1.31) and Schiff (SMD=12.61). In this outcome, 0 studies were trimmed, despite Egger's test showed the presence of publication bias, p-value=0.001. Safe N test required 725 studies to revert

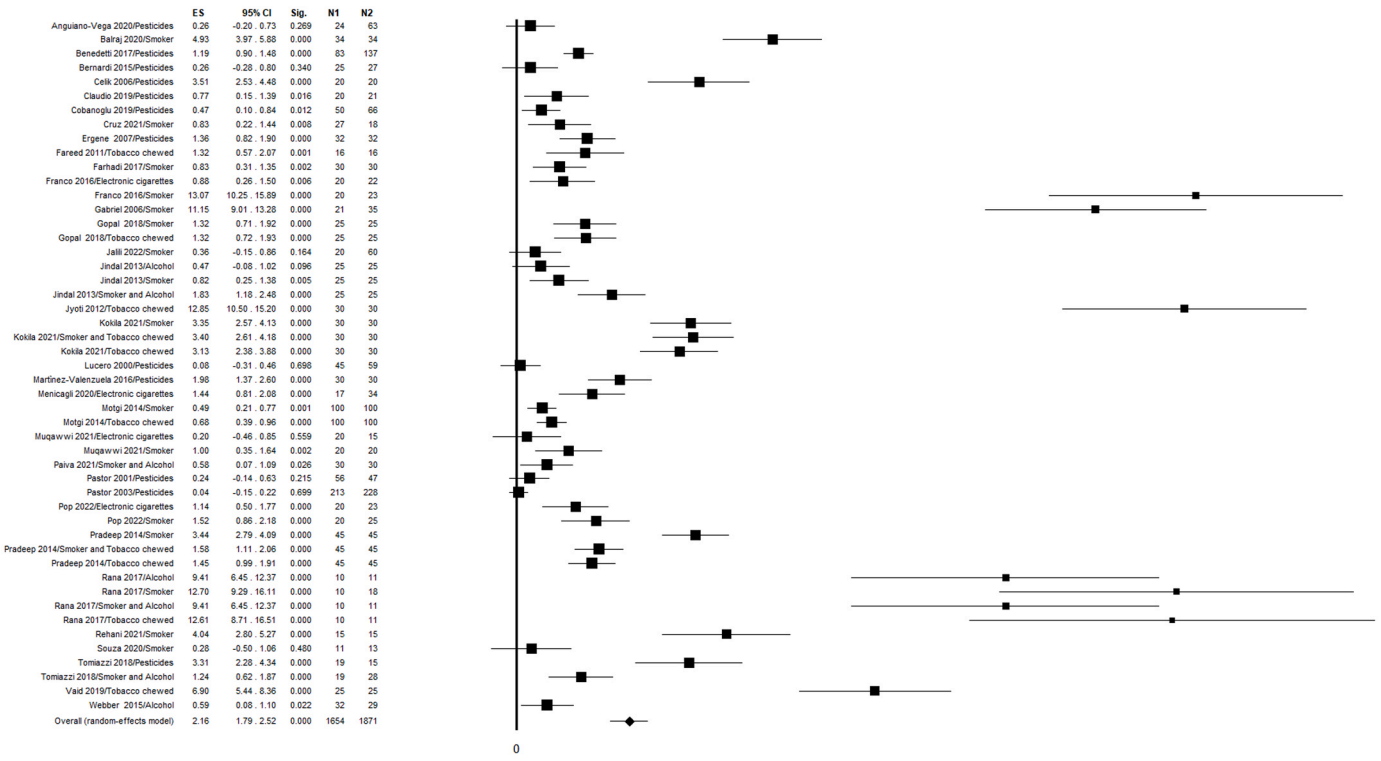

Fig. 2. Forest plot showing a higher number of micronuclei (49 studies including a total of 1654 not exposed vs 1871 exposed participants - SMD=2.16, 95 % CI=1.79–2.52, p-value<0.001, random effects model I^2=95.14).

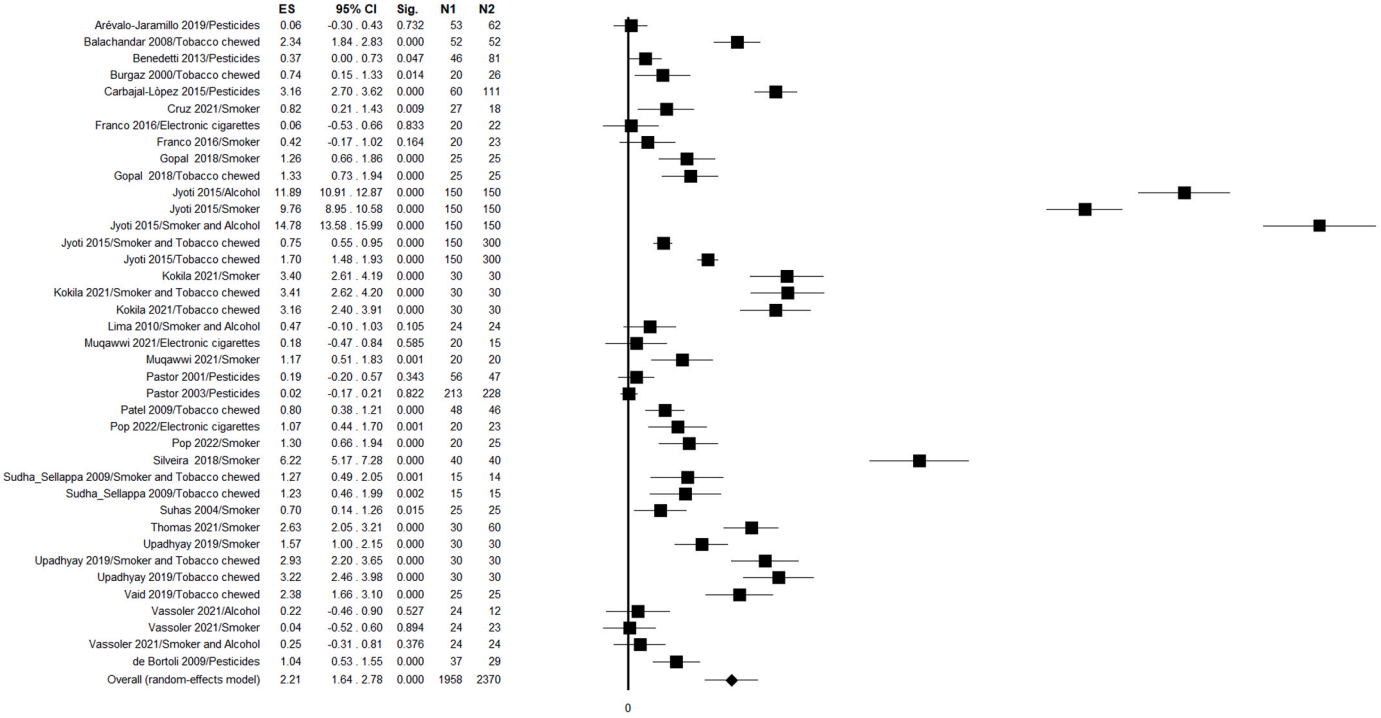

Fig. 3. Forest plot showing a higher number of micronucleated cells (39 studies including a total of 1958 not exposed vs 2370 exposed participants – SMD=2.21, 95 % CI=1.64–2.78, p-value<0.001, random effects model I^2=98.17).

the statistically significant association between higher MNs and chewed tobacco.

– *Effect of chewed tobacco on MNC*: Nine studies [16,17,38,44,49–53] analyzed this issue. The meta-analysis showed that the value of MNC was higher in the chewing tobacco group (SMD=1.84, 95 % CI=1.33–2.36, p-value<0.001), but the heterogeneity was high (I^2=88.62 %). It was observed that publication year impacted on the SMD, with effect size increasing by year (meta-regression p-value=0.036). In subgroup analysis, staining contributed to heterogeneity that decreased to 46.81 % and 35.80 % for Feulgen and PAP,

Table 3
Results of the pooled meta-analyses performed. SMDs and 95 % CI followed by p-values can be observed.

Exposition	Meta-analysis SMD and (95 %CI)	p-value	MN/ MNC assay
Alcohol	2.34 (0.46–4.22)	0.015	MNs
Alcohol	6.05 (-5.39–17.48)	0.300	MNC
Electronic cigarette	0.92 (0.41–1.43)	<0.001	MNs
Electronic cigarette	0.44 (-0.19–1.06)	0.171	MNC
Chewed tobacco	4.14 (2.75–5.52)	<0.001	MNs
Chewed tobacco	1.84 (1.33–2.36	<0.001	MNC
Smoked tobacco	3.04 (2.17–3.91)	<0.001	MNs
Smoked tobacco	2.42 (1.11–3.73)	<0.001	MNC
Smoked tobacco and alcohol	2.25 (0.88–3.63)	0.001	MNs
Smoked tobacco and alcohol	5.14 (-1.22–11.50)	0.113	MNC
Smoked tobacco and chewed tobacco	2.46 (0.69–4.24)	0.007	MNs
Smoked tobacco and chewed tobacco	2.07 (0.68–3.45)	0.003	MNC
Pesticides	1.01 (0.56–1.46)	<0.001	MNs
Pesticides	0.79 (-0.02–1.61)	0.056	MNC
OPMDs	3.03 (1.88–4.18)	<0.001	MNs
OPMDs	1.16 (0.72–1.61)	<0.001	MNC
OSCC	5.86 (3.94–7.79)	<0.001	MNs
OSCC	3.23 (1.73–4.74)	<0.001	MNC

respectively. This resulted in statistically significant differences in SMD by subgroup (ANOVA Q-test p-value=<0.001, SMD_{PAP}=2.90 *vs* $SMD_{Fluorescent}$=1.70 *vs* SMD_{Giemsa}=1.46 *vs* $SMD_{Feulgen}$=1.04). Zero studies were trimmed from the funnel plot and Egger's regression did not indicate the existence of bias (p-value=0.51). Safe N test required 951 studies to revert the significance.

- *Effect of smoked tobacco on MNs*: This outcome was the most studied with 16 studies included in the meta-analysis [16,34,35,39,41,42,44,47,48,54–60]. The results of the meta-analysis showed that patients who smoked tobacco had more MNs (SMD=3.04, 95 % CI=2.17–3.91, p-value<0.001) but the heterogeneity was also high I^2=96.21 %. Subgroup analysis by country did not improve heterogeneity. SMD was the highest in Europe (5.97) compared to America (3.47) and Asia (2.56). Staining subgroup analysis showed similar results with PAP being the most used (6 studies), followed by Giemsa and Feulgen (both 4 studies) and Schiff and fluorescence (both one study). The highest SMD was obtained for Schiff (12.70), compared to Giemsa (6.15), PAP (2.47), and fluorescence and Feulgen (0.82 and 0.70, respectively) (ANOVA Q-test p-value<0.001). Trim and fill trimmed 0 studies in the funnel plot. However, Egger's test showed presence of publication bias (p-value<0.001). However, Safe N test required 1995 studies with opposite outcomes to revert the significance of results.
- *Effect of smoked tobacco on MNC*: Twelve studies [15–17,38,39,41,42,44,55,61–63] studied this outcome. As in the previous case, a high SMD for MNC was observed in smokers (SMD=2.42, 95 % CI=1.11–3.73, p-value<0.001). But the heterogeneity was also high (I^2=98.01 %). When analyzing by country of origin, it was observed that heterogeneity was lowest for Europe (I^2=74.30 %) but only 2 studies were included in this subgroup. It should be noted that statistically significant differences remained only in the Asian subgroup (SMD=3.14, 95 %CI=1.18–5.10, p-value=0.002 – European and American p-values 0.619 and 0.083, respectively). Heterogeneity decreased to I^2=4.86 % in Feulgen staining group, including only two studies. Schiff and Fluorescent reported highest SMD (respectively 6.22 and 4.33, p-value<0.001) followed by PAP (2.09, p-value=0.11), Feulgen (1.04, p-value=0.52) and Giemsa (0.54, p-value=0.68). Despite trim and fill test did not find trimmed studies, Egger's test found presence of publication bias (p-value=0.005).
- *Combined effect of smoked tobacco and alcohol on MNs*: Four studies analyzed the effect of smoked tobacco and alcohol used together on MNs [34,35,64,65]. The meta-analysis showed a higher SMD in patients with these habits (SMD=2.25, 95 % CI=0.88–3.63, p-value=0.001; random effects model I^2=92.27). Two studies were conducted in America and two in Asia. The origin of the studies had an impact in the SMD (ANOVA Q-test p-value=0.028, $SMD_{America}$=0.91 *vs* SMD_{Asia}=4.31). All the studies employed different staining techniques. Egger's test reported presence of publication bias, p-value=0.042, even though zero studies were trimmed in the funnel plot.
- *Combined effect of smoked tobacco and alcohol on MNC*: Only three studies investigated the effect of smoked tobacco and alcohol on MNC [15,38,66]. The result of the meta-analysis showed that patients with these habits did not have significantly higher MNC than the control group (SMD=5.14, 95 % CI=-1.22–11.50, p-value=0.113, random effects model I^2=99.60). In this case, meta-regression showed a decrease in effect size with a higher NOS quality score (p-value=0.008). Moreover, publication bias emerged by Egger's test, p-value=0.006.
- *Effect of combination of smoked and chewed tobacco on MNs*: Only two studies were included in this meta-analysis [16,48]. The results showed how patients using both types of tobacco had a higher number of MNs (SMD=2.46, 95 %CI=0.69–4.24, p-value=0.007, random effects model I^2=93.34).
- *Effect of combination of smoked and chewed tobacco on MNC*: Four studies contributed to this outcome meta-analysis [16,17,38,53]. It was also observed that patients using both types of tobacco had higher MNC values than the control group (SMD=2.07, 95 % CI=0.68–3.45, p-value=0.003). But heterogeneity was also high I^2=95.65 %. Heterogeneity did not decrease when subgrouping for country (Asia=3). However, it decreased to 0 % when considering staining subgrouping (PAP=2). The PAP subgroup reported highest SMD=3.15 (p-value<0.001). Funnel plot with additional trim and fill recorded one trimmed study with a new lower SMD of 1.63 and p-value=0.006 (Egger's test p-value=0.13).
- *Effect of pesticides on MNs*: Twelve studies contributed to this meta-analysis outcome [65,67–77]. The results of this meta-analysis were SMD=1.01, 95 % CI=0.56–1.46, p-value<0.001, random effects model I^2=92.71 %. None of the studies was performed in Asia and subgroup analysis between Europe and America did not find differences (ANOVA Q-test p-value=0.123, SMD_{Europe}=0.72, p-value=0.008 *versus* $SMD_{America}$=1.39, p-value<0.001). When accounting for staining, heterogeneity decreased to I^2=0 % for the fluorescent subgroup (SMD=0.10, p-value=0.227). And differences existed among subgroups (ANOVA Q-test p-value=0.048, SMD_{Schiff}=0.84, p-value=0.13, $SMD_{Feulgen}$=1.09, p-value=0.008, and SMD_{Giemsa}=2.06, p-value<0.001). Publication bias was found by Egger's test p-value=0.009. However, Safe N test required 438 studies to revert main meta-analysis finding.
- *Effect of pesticides on MNC*: For this outcome, 6 studies were pooled in random effects meta-analysis [76–81]. The results showed a SMD=0.79, 95 % CI=-0.02–1.61, p-value=0.056; I^2=96.98 %. Accounting for country subgroup, Europe heterogeneity decreased to I^2=0 % and SMD=0.05 (p-value=0.54). While in the Europe subgroup 2 studies were pooled, for the America subgroup, 4 studies were pooled in a random effects meta-analysis resulting instead in statistically significant difference (SMD=1.15, p-value=0.032, I^2=97.55). It was also observed how staining heterogeneity was null for fluorescent subgroup of 3 studies, resulting in a SMD=0.05 (p-value=0.48). Publication bias did not emerge, Egger's test p-value=0.196.

We will now summarize the most important results regarding the presence of MNs and MNC in the OPMDs and OSCC (Table 3):

- *OPMDs and MNs*: Eight studies contributed to this outcome meta-analysis [82–89]. Random effects model meta-analysis showed

how OPMDs had more MNs than normal mucosa (SMD=3.03, 95 % CI=1.88–4.18, p-value<0.001; I^2=96.27). All the studies were conducted in Asia and SMD varied widely and was statistically different between staining groups (ANOVA Q-test p-value=0.019). In particular, a subgroup meta-analysis was possible for Giemsa and PAP which included 3 studies each. PAP obtained statistically significant results (SMD=5.91, p-value<0.001), but Giemsa subgroup did not find statistically significant differences between OPMDs and normal mucosa (SMD=1.35, p-value=0.21). Considering specific OPMDs, OSMF subgroup meta-analysis was performed including 4 studies (SMD=2.95, 95 % CI=1.49–4.40, p-value<0.001; I^2=94.61). Considering LP, again 4 studies were included (SMD=3.22, 95 % CI=1.40–5.05, p-value=0.001; I^2=94.56). OLP subgroup meta-analysis yielded I^2=0 % and fixed effects model resulted in SMD=1.35, 95 % CI=1.02–1.68, p-value<0.001. Even in this case, Egger's test showed publication bias (p-value=0.027). Safe N test required 780 to revert the statistical combined findings.

- *OPMDs and MNC*: This outcome was explored in 8 studies [82,85,88,90–94] which were pooled in a random effects model (I^2=83.70 %). SMD resulted in 1.16 with 95 % CI=0.72–1.61, p-value<0.001. Only one study was performed in Europe. When the origin of the studies was taken into consideration, Asia subgroup heterogeneity did not differ (I^2=85.97 %) and the elimination of the European study did not impact the SMD (0.95, p-value=0.005). In this case, effect size seemed to be proportional to publication year (p-value=0.078). When accounting for staining techniques, Giemsa meta-analysis yielded null heterogeneity (I^2=0 %). In this case, 4 studies were pooled in the new meta-analysis (SMD=0.70, 95 %CI=0.47–0.93, p-value<0.001, fixed effects model). Subgroup meta-analysis for diverse OPMDs was possible only for LP (SMD=1.17, 95 % CI=0.58–1.76, p-value<0.001; I^2=84.59 %, 5 studies). Egger's test resulted in absence of publication bias, confirmed by 0 trimmed studies in the funnel plot (p-value=0.126). Safe N test required 280 studies to revert this pooled result.
- *OSCC and MNs*: Ten studies focused on this outcome [51,64,84–89,95,96]. Random effects model was employed to pool results in meta-analysis (SMD=5.86, 95 %CI=3.94–7.79, p-value<0.001, I^2=98.24). This result lost statistical significance when considering only the studies performed in America (p-value=0.2). Asia subgroup meta-analysis reported highest SMD=7.28, 95 % CI=4.31–10.25, p-value<0.001). Accounting for staining techniques, there were 5 groups, with PAP being the highest reported (3 studies) SMD=8.92 and p-value<0.001. Feulgen subgroup also included 3 studies, however, SMD obtained a value of 2.07 (p-value=0.31). Publication bias emerged after Egger's test (p-value<0.001). However, 2066 studies with opposite outcomes were required at Safe N test to revert statistical significance.
- *OSCC and MNC*: All the studies included in this meta-analysis were performed in Asia (5 studies) [85,88,90,93,94]. The results of this meta-analysis showed that OSCC presented higher values of MNC than healthy mucosa (SMD=3.23, 95 % CI=1.73–4.74, p-value<0.001, random effects model I^2=95.46). Only two studies were pooled in a staining subgroup, with Giemsa SMD of 2.92 (p-value=0.19). Publication bias was highlighted by Egger's test p-value=0.038. Safe N test required 315 studies to revert meta-analysis results.

4. Discussion

The results of the present study have shown that all the risk factors studied significantly increase the presence of MNs in the oral mucosa with respect to the control group. Similarly, the number of MNs was significantly higher in OPMDs and OSCC than in the control group. When MNC was analyzed, the data were different in some cases. Specifically, no significantly higher results were found for the risk factors alcohol, e-cigarettes, combination of smoked tobacco and alcohol, and pesticides.

The "Human MicroNucleus" project (HUMN), since its foundation in 1997, brought important contributions and improvements in the standardization of MNs assay. In 2007, MNs were quantified in more than 5000 samples showing that in control subjects MNs have a mean of 1.1 over 1000 counted cells [97]. Our meta-analysis has shown a differential increase in SMD based on different risk factors, ranging from 0.92 for e-cigarette to 5.86 for OSCC. Chewed tobacco recorded the second highest SMD, while smoked tobacco, alcohol, a combination of smoked tobacco and alcohol, and OPMDs obtained similar SMDs, ranging from 2.34 to 3.04. This could indicate a higher genotoxic effect of smokeless tobacco compared to smoked tobacco. In patients who chew tobacco, oral mucosa is in constant contact with the chewed tobacco genotoxicant agents [87,98].

Many of OSCCs are associated with different risk factors and sometimes are preceded by OPMDs. These oral lesions can accumulate genetic structural chromosomal aberrations during any stage of cell division. As a result of this, fragments are lost and, therefore, not included in the main nuclei. Once this phenomenon occurs, these fragments are lost in the cytoplasm and can be observed as MNs. Risk factors such as smoked and chewed tobacco, as well as alcohol, are well-known risk factors of genomic instability leading to a higher risk of OSCC. The assessment of MNs assay was proposed as a suitable tool to identify these changes [85]. The highest number of MNs was found in patients with OSCC, with SMD almost twice as high as patients with OPMDs (5.86 *vs* 3.03). Gupta et al. found the highest number of MNs in OSCC, followed by OSMF and leukoplakia [84]. It is noteworthy how OSMF develops in patients who use chewed tobacco [99]. In the present meta-analysis, patients that used chewed tobacco had the second highest SMD (4.14). Thus, chewing tobacco increases genetic damage leading to the development of OSMF, which is one of the most malignant OPMDs [100]. In future studies exploring the effects of chewed tobacco on MNs, the existence of an OSMF must be ruled out. However, in the study by Katarkar et al. when exploring MNs in different OPMDs and OSCC, all of the included patients with OSMF reported a tobacco chew habit, and disease stage was not correlated to MNs [86]. This emerged also in Shah et al. study, where most of patients included in OSMF subgroup presented a history of tobacco. However, in this study, MNs were correlated to OSMF clinical staging [89]. Therefore, in clinical practice, the assessment of MNs could be useful in the follow-up of OSMF [101].

In all the studies included in this meta-analysis, the number of MNs was higher in patients with OSCC than in patients with OPMD. These results were confirmed in two studies [85,88]. Sangle et al. and Shah et al. even correlated MNs to higher OSCC grading [87,89], collecting results similar to previous studies [102,103]. Therefore, the detection of MNs could be useful to assess the malignancy of OPMDs. However, although the number of MNs is assumed to increase during transformation from normal mucosa to advanced OPMD and OSCC, to date no prospective study has been performed to evaluate the utility of using MNs detection to predict malignancy.

Exfoliative cytology followed by MNs quantification is also used to perform genomic toxicology testing for chemical and pharmaceutical product development [104]. There are many studies showing an increase in the number of MNs in different diseases or after exposure to different toxins [105]. In this meta-analysis, e-cigarettes were associated to higher MNs. Despite a social perception of safety in the use of e-cigarettes [106,107], the findings of the present study support the genotoxic role of e-cigarettes [108,109]. However, comparative studies found relatively lower MNs in e-cigarettes than in smoked tobacco [39,41], and also this meta-analysis obtained similar results (SMD 0.92 *vs* 3.04). This finding might reflect the amount and the activity of genotoxicants produced in e-cigarettes compared to smoked tobacco. It was found that many compounds had lower concentrations in e-cigarettes compared to smoked tobacco [109,110]. While e-cigarettes might be proposed as substitutes for smoked tobacco during smoking cessation, these should not be considered as a healthy option, and the aim should

always be quitting smoking [107]. While more studies are needed to ascertain the effects of e-cigarettes on human health, MNs assay might be a useful tool in monitoring this population.

Similar SMD was reported in subjects exposed to pesticides (1.01). To date, it is unclear the role of pesticides in oral cavity carcinogenesis [111], and previous reports led to inconsistent results [67,112]. This variability could depend on population study. First, it seems to exist a relation between pesticide exposure time and MNs with impact on gender bias related to working time for males compared to females. Heterogeneity among reports might also follow a seasonal bias, with differences in working hours among seasons [72]. In last instance, in the literature emerged a low frequency of personal protective equipment use during handling of pesticides, which might vary among countries and regulamentation [75]. Results from this meta-analysis indicate higher MNs in subjects exposed to pesticides. However, considerations about development of oral pathologies were not considered. Future studies should consider the above points. In addition, prospective studies should be conducted to assess whether pesticides are associated with the development of OPMDs and OSCC.

Despite promising results found in this meta-analysis, translational application into clinical practice has limitations. These include intra- and inter-individual heterogeneity, both from the patient' perspective and from the methodological adequacy in the quantification of MNs and in the way the data are offered. HUMN has worked to find out the origin of the heterogeneity. Their results showed that this heterogeneity is due to demographic and methodological variables in the different laboratories. However, 25 % of this variability remained unexplained [105, 113].

Most of the subgroup meta-analyses performed showed high heterogeneity, even taking into account the origin of the studies and staining techniques. Of the 5 staining techniques used, Giemsa and Feulgen were the most used. However, Giemsa and PAP accounted for 70.6 % (24/34) of staining techniques used in Asian studies, whereas the American and European studies used the Feulgen technique more, followed by Giemsa at 68.4 % (13/19) and 53.8 % (7/13), respectively. These differences may be due to the cost of staining protocols. In any case, no studies have been performed to evaluate the cost-effectiveness of the different techniques to detect MNs. On the other hand, some studies showed modifications in the staining protocols which may further contribute to heterogeneity. For example, Schiff was used alone [35,61,96] or in combination with Light Green [68,72]. Similarly, 3 studies included changes in the Giemsa protocol, performing May--Grünwald- [41,53] and Leishman staining [83]. The Fluorescent subgroup was the most heterogeneous, encompassing four different techniques (Ethidium Bromide, Diff-Quik, DAPI and Acridine Orange). DAPI was the most common (5 studies) [76,77,86,92,93]. We should note that the most recent guidelines on the protocol for detecting MNs [105] have suggested that it is best to use Feulgen and Schiff's reagent and Light Green to perform MNs detection studies in oral mucosa. They also indicate that it is necessary for the investigator quantifying the MNs, to be blinded to patient characteristics. It is noteworthy how many of the included studies did not follow these recommendations [105].

Feulgen staining yielded the lowest SMDs in many of the meta-analyses performed. In a comparative study by Grover et al. between Feulgen, PAP and hematoxylin/eosin techniques for the detection of MNs, the Feulgen technique obtained the lowest counts. This may be because the Feulgen technique is a DNA-specific stain. However, meta-analyses performed showed higher SMDs for the PAP technique. PAP is a non-specific stain that could yield misinterpretations and miscounts of nuclear abnormalities [114]. Few studies have been performed to address the variability of results depending on the staining protocol. Casartelli et al. compared Giemsa, Feulgen, Hoechst 33342, and propidium iodide [115]. These authors recommended Hoechst 33258 while noting that the other stains were unsatisfactory. It is noteworthy how none of the included studies in this meta-analysis employed Hoechst 33258. Furthermore, the comparisons of Casartelli et al. did not include controls, and the samples were collected from heterogeneous groups. On the other hand, Nersesyan et al. found that MNs detection was highly depended on staining methods when quantifying MNs between smokers and non-smokers [116]. The results of our meta-analysis found overall higher MNs values in smokers (3.04, 95 % CI=2.17–3.91, p-value<0.001). But there were differences in the results if we consider the type of stains used, such as Schiff (12.70), Giemsa (6.15), PAP (2.47), fluorescence (0.82) and Feulgen (0.70) (ANOVA Q-test p-value<0.001). Although higher rates of MNs were identified in smokers with the non-specific DNA stains, Giemsa and May-Grünwald, no significant differences were observed with acridine orange, DAPI, and Feulgen in the study of Nersesyan et al. [116]. Our meta-analysis may support the results of Nersessyan et al., even though the number of studies included in each subgroup may be insufficient.

More variables should be considered as sources of heterogeneity in this meta-analysis, including sample collection location. Guidelines suggest that samples should be collected in the right and left buccal mucosa [105]. However, our inclusion criteria did not take these criteria into account. Mohanta et al. found that the buccal mucosa had the highest percentage of MNs, followed by the tongue, floor of the mouth and palate. However, the lowest results were for the alveolus and gingiva. It is expected that this variety in locations may be due to the difference between the cells in the different locations, since in the oral mucosa keratinization is different. There is less keratinization in the bottom of the mouth, while the levels are higher in the lips [116,117]. It should be noted that some studies highlighted that the method used to collect oral mucosa cells may represent a source of technical heterogeneity. Previous authors have shown that the rigor and vigor in collecting the sample may also affect the frequency of detection of oral MNs [118].

Using the buccal mucosa as the location of choice may be the correct way to evaluate MNs in systemic pathologies or toxicants exposure (tobacco, alcohol, or pesticides). But we believe that this protocol should be modified when we want to evaluate the presence of MNs in oral lesions such as OPMD and OSCC. It is logical to think that in these cases it is better to collect the sample from the area affected by OPMDs or OSCC. However, it is difficult to determine where to take the sample in those investigations that aim to evaluate oral carcinogenesis and field cancerization processes.

It is noteworthy how in certain studies there were slight differences in the results of MNs between males and females. On the other hand, in OSCC samples it was observed that the results were lower in younger patients [119]. Age, sex, and oral cavity subsites may represent an additional source of heterogeneity and bias in reporting results, and to date there is little evidence addressing these points. For example, it takes 14–24 days for a stem cell to divide and progeny to traverse the full thickness of the epithelium (turnover time) [120,121], and this turnover is expected to slow down in older patients [121]. On the other hand, an increase in genomic instability and a decrease in DNA safeguarding machinery with aging is to be expected [122]. Hopf et al. observed a multiplicative slope of 14 % for every 10 years of age for buccal cells [123]. Similarly, Ferraz et al. reported that the frequency of MNs was significantly higher in older individuals [124]. Taken together, these variables may explain additional heterogeneity across studies.

Also, the heterogeneity of the results may be due to the scoring used to determine MNs. Since nuclear division is necessary for MNs, the scoring should logically be limited to the basal cell layer, as this is the regenerative layer where nuclear division takes place. On the other hand, the change in cell division kinetics happening in the context of the oral mucosa exposed to genotoxic agents or in OPMDs and OSCC could have an impact on how MNs are expressed in these cells. Normal differentiated cells that are the daughter cells of the basal layer and have already undergone nuclear division are another possibility for MNs scoring. Although there are no data to support or reject this assumption, the counterargument for normal developed cells is that they may lose MNs during the differentiation process [118]. In this study, we observed different ways of reporting results among the included studies. We found

studies that showed the results in both MNs and MNC. Moreover, the results were expressed over 1000 counted cells in 55.2 % of studies, and in other cases, they ranged from 50 to over 3000 total cells counted. This is also an important point to discuss, as guidelines support that the number of MNs should be reported after scoring a minimum of 2000 differentiated cells. However, no indications for MNC were given [125].

All the risk factors included in this meta-analysis were found to be associated with increased MNs, in particular chewed tobacco and smoking. Also, MNs were higher in patients with OPMD and OSCC than in controls. Despite the large number of included studies, a high heterogeneity emerged among studies and subgroup analysis revealed, in some cases, discordant results. This may be attributed to several limitations of the MNs assay itself. We strongly recommend that future studies follow the protocol published by Bolognesi et al. [105]. Moreover, the expert panel should focus on evaluating the cost-effectiveness of protocols to determine MNs, considering the complexity of oral MNs pathophysiology and the integration of automatization into the assay as well as the counting execution. In the future, the application of machine learning and deep learning may show promising benefits at this point.

Author contributions

MEPI contributed to the study design and drafted the manuscript. VCAC, FFVS, FP, SG, and FS contributed to the acquisition of data, statistical analysis, and manuscript writing. VCAC, RMLP, KZ, LLM, and AILP contributed to the study design, writing, and reviewing of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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The graphical abstract was created with BioRender.com.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mrrev.2024.108508.

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