Reviewing the oral carcinogenic potential of E-cigarettes using the Bradford Hill criteria of causation

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Abstract: The past decade has seen a surge in the use of e-cigarettes, which has prompted the medical community to assess any associated potential health hazards. A major concern was the risk of cancer. Chemical analysis of e-cigarettes has shown the presence of volatile organic compounds with the potential for carcinogenicity. Comparative toxicology analysis has shown e-cigarette to have relatively lower dosages of toxins than conventional combustible cigarettes. Based on comparative analysis, e-cigarettes have been increasingly advocated as a safe alternative to conventional cigarettes. It is vital to recognize that presence of relatively lower toxin level does not preclude carcinogenic potential. The nicotine present in the e-cigarette was presumed to be the major cytotoxic agents, thus nicotine-free e-cigarette was considered as inert. On the contrary, experimental studies on oral cell lines have shown DNA strand breaks on exposure to e-cigarette vapors with or without nicotine. In addition, dysregulations of genes associated with carcinogenic pathways have also been demonstrated in oral tissues exposed to e-cigarette vapors. Despite alarming molecular data, the oral carcinogenic potential of e-cigarette remains unclear, which can be attributed to the lack of long-term prospective and large-scale case-control studies. As e-cigarette users often have other well-established risk factors (conventional cigarette smoking, alcohol, etc.) as associated habits, it is difficult to assess e-cigarette as an independent risk factor for oral cancer. Thus, the present manuscript aims to review the published literature using the Brad Ford Hill criteria of causation to determine the oral carcinogenic potential of e-cigarettes.

Keywords: Carcinogens; cigarette smoking; electronic nicotine delivery systems; mouth neoplasms; volatile organic compounds

Introduction

Tobacco is a well-established independent risk factor for oral cancer (1-7). Tobacco when consumed in combination with alcohol, increases the oral cancer risk exponentially (8-13). Tobacco is mainly consumed by smoking or chewing. The most common forms of smoking tobacco are cigarettes, pipes, shisha, and bidis, while pan masala, mawa, gutka, zarda, toombak, khaini, Shammah and naswar represent the common forms of smokeless/chewed tobacco (14-17). Given the overwhelming evidence of cancer risk associated with both smokeless and smoking form of tobacco, large scale restrictions are sanctioned by health agencies all over

the globe (18,19). Tobacco products are mandated to carry warnings indicating the health hazards associated with their use. Like alcohol, the sale of tobacco products has been forbidden to individuals under the legal age (20). Further, smoking in public is restricted to prevent health hazards associated with secondary smoking (21,22). Governments have constantly increased the tax on tobacco products, to increase its base price with the intention of reducing its sales (23,24). Health agencies have regularly conducted tobacco cessation and awareness programs to deter present and future tobacco users (25,26).

Based on the above-mentioned large-scale strategies employed by government and health agencies, one may think that the tobacco industry may have significantly reduced its productivity due to the decreasing sales. On the contrary, the tobacco industry has found innovative means to rejuvenate its sales. The major strategy employed by the tobacco industry included the introduction of newer products, marketed to be safe alternatives to conventional tobacco products (27,28). One such innovation is the electronic cigarette (e-cigarette). The introduction of e-cigarettes ensured a steady product flow. Unlike most commercial tobacco products, there is not much information as to the effects of e-cigarettes on health, especially with respect to cancer. Thus, without sufficient evidence, implementing anti-tobacco regulations is problematic. Sustained widespread marketing of e-cigarettes as a safe alternative to conventional tobacco products has large scale repercussions. In addition to conventional smokers shifting to e-cigarettes, even previous non-smokers are predisposed to vaping (28,29). E-cigarettes have been advocated along with nicotine patches as conventional tobacco replacement products, which in turn created a false sense of safety among its users (30-32).

Given the increasing global use of e-cigarettes, researchers have focused on understanding their short and long-term effects. Examination of the components of various brands of e-cigarettes has shown the presence of several well-established carcinogenic agents (33-41). In addition to this, studies have also investigated the molecular changes induced by e-cigarettes on the oral mucosa. The results have been alarming with reports ranging from reduced anti-oxidants levels, gene dysregulations to DNA strand breaks (42-46). There were a growing number of oral health hazards associated with e-cigarettes, with potential links to oral cancer (42-63). To implement preventive measures, conclusive evidence of e-cigarette induced oral carcinogenesis is required. Thus, the present review uses the Bradford Hill criteria of causation to assess the oral carcinogenic potential of e-cigarette based on published literature. Bradford Hill's criteria of causation consist of a total of 9 parameters (64-66). All 9 parameters must be satisfied to infer a causal designation to e-cigarette for oral cancer.

Strength of association

Epidemiological studies are considered to be preliminary evidence for a causal relationship. Based on the prevalence of a potential risk factor in a specific disease, a hypothesis for causal inference is generated (64). The major limitation in evaluating the strength of association in multifactorial diseases like cancer is the presence of confounding factors (66). E-cigarette users are often current or past users of conventional cigarettes and have also shown to exhibit other known risk factors such as alcohol consumption (67). Thus, in such cases, the strength of association (statistical significance) from the epidemiological data must be evaluated only after accounting for the potential confounders. Longitudinal observational studies on e-cigarettes have a relatively shorter follow up period, given that research focus on e-cigarette began to rise just a decade ago (60,61). Most observational studies on e-cigarettes involve comparative analysis of toxic products released and their potential health impacts (52,68-73). The comparison groups have ranged from only e-cigarette users, only combustible cigarette users, to dual users (both e-cigarette and combustible cigarette users) (52,68-70). In some studies, instead of having multiple comparison groups, the comparison is made in the same individual as their habit is changed from conventional cigarette to e-cigarette (71-73). Although most of these observational studies varied in their methodologies, the common consensus was that the toxic product dosage and health hazards were relatively less in e-cigarettes than combustible cigarette users and dual users (52,68-70). In studies involving a shift in smoking habits, health hazards, and toxicity decreased after replacing combustible smoking with e-cigarettes (71-73). Some of the assessed toxic products are proven or potential carcinogenic agents, and thus although their dosages have shown to be less in e-cigarette users than combustible cigarette users, they still could pose a cancer risk.

Table S1 summarizes the published data on the *in-vitro*, *in-vivo*, and clinical assessment of e-cigarettes, along with their various comparison groups (74-84). *In-vitro* studies on e-cigarettes have largely focussed on assessing the

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carcinogenic constituents of the cigarette and analyzing their effects on the oral cell lines. The mutagenic potential of e-cigarette was examined by Thorne *et al*. (74) on Salmonella typhimurium strains TA98 and TA100. The results showed that the aerosol collected matter from the e-cigarette did not have any mutagenic effect in either of the test strains. In comparison, the conventional cigarette (3R4F cigarette) exhibited mutagenicity within 24 hours.

Although most of the above-mentioned studies have shown that the toxicity of e-cigarette is substantially lower than conventional cigarettes, it is not clear if the reduced toxicity of e-cigarette could be carcinogenic to the oral mucosa. In addition, it was believed that the nicotine in the e-cigarette vapor was the major cause for toxicity, and thus a nicotine-free e-cigarette could potentially be inert. To test this hypothesis, Yu *et al*. (43) examined the effects of vapor from e-cigarettes with and without nicotine on cell lines of normal oral cells and head and neck squamous cell carcinoma. The results showed that irrespective of the presence or absence of nicotine, the vapors from the e-cigarettes induced cell death through apoptosis or necrosis and caused DNA strand breaks. Evidence of DNA strand breaks from Yu *et al*. study was supported by Kadimisetty *et al*. (39), and Holliday *et al*. (44). Although it is unclear if the DNA strand breaks were sufficient for carcinogenic transformation, there is sufficient evidence to indicate that e-cigarettes with or without nicotine cannot be considered to be inert.

Unlike a combustible cigarette, there is a lack of literature on the effects of e-cigarette on the oral mucosa. Tommasi *et al*. (42) compared the gene regulation and pathways of molecular pathogenesis on the oral cells of users of only conventional cigarette and only e-cigarette with non-smokers. The results showed that similar to conventional cigarette smokers, even e-cigarette users showed dysregulation of genes involved in carcinogenic pathways. Although the e-cigarette has shown to be relatively less harmful than a combustible cigarette, most of these studies just evaluated the levels of toxic exposure. Studies analyzing the effects of these toxins on the oral cells including that of Yu *et al*. (43), Holliday *et al*. (44) and Tommasi *et al*. (42) provide a strong case for the carcinogenic potential of e-cigarettes. The evidence of DNA strand breaks *in-vitro* cell line study and dysregulation of genes involved in carcinogenesis provides the necessary strength of association for considering e-cigarette to be a potential oral carcinogenic risk factor (42-44). Further studies required to strengthen the association would include

decoding the genomic, proteomic, and secretome profile of oral keratinocytes exposed to e-cigarette vapors. In addition, longitudinal studies including patients with only e-cigarette habits with no prior history of any other oral cancer-associated risk habits could aid in assessing e-cigarettes as an independent risk factor.

Consistency

Consistency according to Bradford Hill refers to similar results obtained from different techniques in different populations that will enhance the causal relationship (64). As mentioned above, the preliminary evidence for the cause is derived from epidemiological data. The advent of molecular biology has allowed data integration between preliminary epidemiological evidence with molecular carcinogenic pathways, which provides comprehensive evidence for a causal relationship. Although individual case reports/series do not carry the same weight as long term prospective or large-scale case-control studies, in the absence of large-scale epidemiological data, preliminary evidence from case reports could be used to formulate a hypothesis. Nguyen *et al*. (48) reported 2 cases of oral squamous cell carcinoma (OSCC) associated with the chronic use of e-cigarettes. In both cases, there has been no other associated habits/ factor.

Despite the lack of convincing clinicopathological evidence, studies have shown considerable molecular data. Tommasi *et al*. (42) observed upregulation of 857 transcripts (74.4%) and downregulation of 295 transcripts (25.6%) in oral epithelium exposed to e-cigarette vapors. Molecular pathways and functional analysis revealed a 62% association of e-cigarettes with oral cancer. Wnt/Af pathway was indicated as a major route for E-cigarettes induced oral carcinogenesis. Gene dysregulation and the DNA strand breaks induced by e-cigarettes on oral cells provide additional molecular evidence for the toxic nature of e-cigarettes on the oral mucosa (39,43,44). In addition, e-cigarette vapors have shown to contain variable levels of aldehyde carbonyls which are known to cause oxidative stress, DNA adduct damage, stress-induced cellular senescence which in turn have shown to induce carcinogenesis (45,46). E-cigarette exposure mediated upregulation of RAGE increases prostaglandins and COX2 in gingival epithelial cells have also been implicated in carcinogenesis (85). Sundar *et al*. (45) reported e-cigarette induced oxidative stress, carbonyl stress, DNA damage, HDCA 3 reduction, increased inflammatory response in gingival epithelial cells which could potentially lead to

malignant transformation. Based on the above data, there is consistent evidence for e-cigarette induced carcinogenicity to the oral mucosa.

Specificity

Due to the lack of long-term clinical studies on the use of e-cigarettes, there is a scarcity of direct evidence for oral malignant transformation. The added difficulty in specifically associating e-cigarettes to oral cancer is due to the wide range of newer generations of e-cigarettes being produced. The newer generation products have different designs patterns from existing models, with adjustable voltages and flavorings (86). In such cases, the best way to assess the carcinogenic potential of the different e-cigarette designs would be to assess the common ingredient present in their vapor. Over 115 volatile components have been found in e-cigarette aerosol (87). Potential e-cigarette carcinogens include metals (cadmium, chromium, etc.), carbonyls (acrolein), propylene oxide, and especially flavoring additives. The concentration of chemicals depends on many factors. Higher voltage is associated with a 10-fold increase in the release of inflammatory cytokines, which in turn have shown to increase the reactive oxygen species (ROS) causing significant DNA damage (88). Flavoring chemicals like vanillin, ethyl vanillin, ethyl maltol, and menthol and toxic aldehydes (acetaldehyde, formaldehyde) have been found to cause DNA strand breaks as demonstrated by increased comet tail length and γ-H2AX foci numbers. These effects are regardless of nicotine concentrations and are observed in both short term and long term (40,43). It is increasingly accepted that ROS formed due to the vaporization of such liquids are probably responsible for carcinogenesis (37,46). Another laboratory assay showed that refill solutions were cytotoxic, especially to stem cells, irrespective of voltage (41). A rat model study showed phase I carcinogen bio-activation and DNA damage at both chromosomal and molecular levels (50). Based on the presence of known carcinogenic agents, there is sufficient proof for specificity. Future studies must focus on long term prospective studies on individuals with only e-cigarette habits. Malignant oral mucosal changes in such individuals would in turn provide additional support for specificity.

Temporality

The scarcity of longitudinal studies on e-cigarettes renders

temporal causation analysis a tedious job. An alternative approach would be to analyze the association between e-cigarettes and oral potentially malignant disorders (OPMDs) (66). Since there is an abundance of literature and conventional acceptance regarding the temporal link of OPMDs and oral cancer, an analysis of e-cigarette and OPMDs would hold relevance (89). However, studies with OPMDs and e-cigarettes are also scarce. A Google Scholar and PubMed search using he terms "oral potentially malignant disorders/premalignant lesions and e-cigarettes" did not reveal any relevant results. A possible link between e-cigarette use and oral submucous fibrosis (OSMF) was discussed by Javed *et al*. (53). The combined exposure of nicotine and arecoline (from associated chewing of areca nut) was shown to induce keratinocyte senescence and fibroblast proliferation. Based on the results, the authors implied that malignant transformation of OSMF may be further induced/accelerated by nicotine from the e-cigarettes.

Apart from OPMDs, there are other less established oral mucosal changes with an increased risk for oral cancer. Some of these oral mucosal changes have shown to be induced by the components of e-cigarettes which in turn could be used as evidence for temporality. Renne *et al*. (54) assessed the effect of aerosolized glycerol inhalation in rats. The exposure to propylene glycol resulted in the development of squamous metaplasia of the epiglottis. A similar study in rats revealed that exposure to acrolein, acetaldehyde, and formaldehyde caused sensory irritation (55). A crossover trial by Bullen *et al*. (90) reported that the most frequent adverse effects of e-cigarette included mouth and throat irritation. Persistent local irritation may result in pathological mucosal reactions that may predispose to the development of potentially malignant oral disorders. A systematic review by Farsalinos *et al*. (57) found that even though such clinical adverse effects have been found, they were considerably lower than conventional smoking. Lack of carcinogenic evidence on e-cigarettes was supported by data from another systematic review by Pisinger *et al*. (58). Based on the 76 studies analyzed in their systematic review, Pisinger *et al*. (58) stated that there were no significant adverse effects in e-cigarette smokers that might lead to oral cancer. Although there is evidence to indicate that e-cigarette contains potentially carcinogenic agents, its presence seems to be relatively lower than conventional cigarettes as supported by the lack of oral cancer association from the systematic reviews (57-59). On the contrary, there is also evidence to show e-cigarette induced molecular

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changes (39,42-44) could potentially lead to cancer. As e-cigarettes are a recent innovation, it might be too soon to comment on a temporal association with cancer, but the evidence of molecular dysregulation and DNA strand breaks cannot be unnoticed. Long term perspective research in individuals with only e-cigarette habit is needed for gaining temporal association for the e-cigarette with oral cancer.

Biological gradient

In epidemiology, increased exposure of a potential risk factor results in an increased incidence of the disease, which in turn can be used for establishing a causal inference (64). In multifactorial diseases like cancer, the dose-response curve is non-linear due to the presence of confounding factors (66). Biological gradients are difficult to characterize in such cases due to individual susceptibility (genetic predisposition) and synergistic or antagonistic effects of cumulative exposures. In cases of oral cancer with e-cigarette usage, there is often a concomitant use of other known risk factors including combustible cigarettes, thus, estimating a biological gradient in such cases would not reveal the true dose-response pattern. Modern analytics have shown that molecular changes can occur even in the low-dose range of environmental chemical exposures, although these changes may not lead to observable disease. Thus, even though studies have shown e-cigarette to have relatively lower dosages of toxic components than conventional cigarettes (52,68-73), even these lower dosages of toxin could induce molecular changes in the oral mucosa with no apparent clinical change. Future studies on e-cigarette exposure must include a complete molecular panel of proteomic, genomic, and, secretome analysis to elicit the subtle changes induced by e-cigarettes. At present, there is a paucity of research related to the direct physiological effect of the e-cigarette vapors on oral tissues. Apart from studies demonstrating e-cigarette induced DNA strand breaks (43,44) in oral cells and eliciting the presence of few dysregulated genes (39,42), there are no comprehensive biological gradient studies to elicit a dose-response relationship between e-cigarettes and oral cancer.

Biological plausibility

For a potential causal agent to be implicated in a specific disease, there must be a defined etiopathogenesis (64). Since the advent of molecular biology, several carcinogenic pathways have been delineated for several cancers,

including oral cancer. Thus, establishing an e-cigarette mediated pathway for oral carcinogenesis would aid in supporting a causal inference. The first step in assessing biological plausibility would be to establish the presence of carcinogens in e-cigarettes. As mentioned earlier, a chemical analysis of e-cigarette aerosol has revealed more than 115 volatile components (87) including metals (cadmium, chromium, etc.), carbonyls (acrolein), propylene oxide, flavoring additives. The second part would be to establish that the carcinogenic compounds come in contact with the area of interest and that the area of interest has reported evidence of cancer. Over 90% of all human cancers are of epithelial origin (91,92) and the oral epithelium is the first site of exposure to carcinogens present in e-cigarette vapor. The third part would be to confirm that the agents released by the e-cigarette vapors have a detrimental effect on the oral mucosa at the clinical, histopathological and molecular levels. At a clinical and histopathological level, there is not much evidence, other than that e-cigarette users develop oral mucosal changes which in turn may carry a risk for oral cancer (49,56,93). In contrary to clinicopathological evidence, there is a relatively greater number of studies assessing the molecular changes induced by e-cigarettes in the oral cells. Several studies including that of Yu *et al.* (43) have confirmed the e-cigarette induced DNA strand breaks in oral cells. In addition, whole transcriptome analysis of oral cells from e-cigarette users, have shown deregulation of key genes, the majority of which converging on cancer-related pathways and functions (94-96). The final part of biological plausibility would require decoding the e-cigarettes mediated molecular pathways of oral carcinogenesis. Based on the above evidence, it is biologically plausible that e-cigarette causes molecular changes in oral cells, but it is not clear if the changes lead to malignant transformation.

Coherence

For causal inference, the risk factor in question must be associated with the disease consistently across studies with different research designs (64). Thus, data from epidemiological studies must be coherent with data from other research design including *in-vivo*, *in-vitro* experimental studies. The E-cigarette has shown to have carcinogenic components and their vapor on exposure to oral cells has shown to cause changes DNA strand breaks and gene dysregulations (33-45). DNA double-strand breaks are the most lethal form of DNA damage, and if left

unrepaired, they can result in chromosomal rearrangement and could potentially lead to carcinogenesis. The major cause of conflict in the evidence for an e-cigarette mediated oral carcinogenesis is the studies eliciting that replacing the conventional combustible cigarette with e-cigarettes reduces the overall toxicity (52,68-73). Although e-cigarette carries a lower toxin level than a combustible cigarette, even the lower levels of e-cigarettes toxins could potentially carry an oral cancer risk. Based on the evidence, there is coherence in the evidence for the carcinogenic potential of e-cigarettes.

Experiment

The mere presence of a potential risk factor in a disease entity does not confer a causal status. It is possible that the disease predisposes accumulation of the agent in question, which could, in turn, explain its high prevalence in the disease entity. Thus, even a strong epidemiological association is not sufficient for causal inference. Conclusive evidence would require experimental studies exposing the potential risk factor to a physiological tissue and inducing the disease status. In addition, removal of the potential risk factor must result in regression of the disease, unless the disease has progressed to an irreversible state as in malignancy. Although the presence of carcinogenic components in e-cigarettes and their detrimental effects (DNA strand breaks and gene dysregulation) (39-43) on oral cells provides considerable evidence of carcinogenicity, there are no longitudinal studies that have shown that e-cigarette cessation reverses or reduces the disease state. Further, in multifactorial diseases like cancer, the associated e-cigarette might be a coincidental factor and the other known risk factors (combustible cigarettes, alcohol, etc.) could have been the causal agent (17,27,67). Thus, clinical experimental studies need to be specific in their inclusion criteria wherein patients with only e-cigarette history are included. Such patients should be observed for carcinogenic molecular changes during and post-cessation of e-cigarette usage.

Analogy

In circumstances where accurate measurement of the confounding factors and quantification of exposure may be difficult, clear cut analogy could shed light to of evidence for potential causative factors, that are otherwise considered as weak association, provided such factors possess similar property/condition as that of the factor with a stronger association with the disease (64). In other words, when there is enough evidence to substantiate causal relationship with a specific agent and disease, a second agent that is similar in some aspects with weaker evidence of association with the disease can also be established as a casual association. It is well-established that combustion of tobacco in regular cigarettes releases toxins and carcinogenic molecules which can cause oral cancer. In comparison, e-cigarette also possesses such carcinogenic agents, although at a lesser concentration than combustible cigarettes (52,68-73). Exposure of oral cells to combustible cigarettes have shown to cause carcinogenic molecular changes. Similar molecular changes (DNA strand breaks and gene dysregulation) have also been observed in oral cells exposed to e-cigarette vapor (39-43). The additional information, which is missing in e-cigarettes studies and, which confirms the carcinogenicity of combustible cigarette is molecular data delineating the various oral carcinogenic pathways and clinical data eliciting reduction of potentially malignant lesions following habit cessation. Applying the concept of analogy, it can be hypothesized that e-cigarettes having carcinogenic agents and capable of inducing DNA strand breaks (43,44) and gene dysregulation (39,42) in oral cells could also be implicated in oral cancer.

Conclusions

Lack of long term prospective and large-scale case-control studies is a major limiting factor in assessing the association of e-cigarettes with oral cancer. In addition, comparative analysis suggesting a relatively lower toxic level in e-cigarette than combustible cigarettes, in turn, creates a misconceived notion on the safety of e-cigarette use. It is important to acknowledge that a relatively lower toxic dosage, does not mean it is risk-free. Evidence of the presence of carcinogenic agents in e-cigarettes and their vapor inducing DNA strand breaks and gene dysregulation is compelling evidence, but not sufficient to infer a causal relationship. Experimental studies are needed to assess if the relatively lower dosage of toxin released by the e-cigarette is capable of inducing malignant transformation in oral epithelial cell lines and mouse models. Further delineating the molecular pathway of e-cigarette induced oral mucosal changes could provide valuable insight into e-cigarettes' oral carcinogenic potential and would also aid in identifying diagnostic markers and therapeutic targets. In addition, longitudinal studies with

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longer follow up periods are needed, wherein the included cases must be only e-cigarette users, with no history any other associated risk factor. Evidence of malignant mucosal changes in such cases would provide the necessary clinical evidence for a causal association, although, still genetic predisposition would be a confounding factor. To conclude, based on the evidence from the published literature, the e-cigarette can be considered as a potential risk factor for oral cancer due to the presence of carcinogenic components and due to their ability to induce detrimental changes to oral cells, although there is insufficient evidence for causal inference.

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Table S1 Summary of the published data assessing the *in-vitro*, *in-vivo*, and clinical effect of e-cigarette and comparative groups

S. No	First authors name/year/country (reference)	Aim	Comparative groups	Results
1	Taylor/2016/UK (75)	*In-vitro* assessment of the cellular stress response of the human bronchial epithelial cells to the comparative groups	E-cigarette aqueous aerosol extract (AAE); tobacco smoke AAE	E-cigarette AAE concentration up to 0.5 puffs/mL did not elicit any cellular stress response. Tobacco smoke AAE concentration of 0.063 puffs/mL elicited cellular stress-related responses
2	Misra/2014/US (76)	*In-vitro* assessment of toxicity induced in human lung epithelial carcinoma cells and the Chinese hamster ovary cells by the comparative groups	E-liquids and pad-collected aerosols of e-cigarettes; pad-collected smoke condensates of tobacco cigarettes; aqueous extracts of smokeless tobacco (SLT); aqueous extracts of nicotine replacement products (NRP)	E-cigarettes, SLT and the NRP did not show any significant toxicity, even at doses 100-fold higher than tobacco cigarette smoke. Tobacco cigarette smoke showed significant toxicity
3	Breheny/2017/UK (77)	*In-vitro* assessment of the biological effects on the Bhas 42 mouse fibroblast cells by the comparative groups	Aerosol collected matter (ACM) of electronic cigarette (Vype ePen); total particulate matter (TPM) of cigarette (3R4F)	The tumor-promoting particulate matter was negative in the e-cigarette's ACM up to a concentration of 120 mg/mL (77 ng/mL nicotine). The tumor-promoting particulate matter was positive even at a low concentration of 6 mg/mL TPM (12.5 ng/mL nicotine) for 3R4F's TPM
4	Yu/2016/US (43)	*In-vitro* assessment of the short-term (48 hours) and long-term (8 weeks) effects of the comparative groups on the normal epithelial and head and neck squamous cell carcinoma	Nicotine-containing vapor from e-cigarettes (e-cigarettes brands V2 and VaporFi); nicotine-free vapor from e-cigarettes (e-cigarettes brands V2 and VaporFi)	E-cigarette vapors with and without nicotine reduced the viability and clonogenic potential of the cells while increasing cell death (apoptosis and necrosis), comet tail length and DNA strand breaks
5	Taylor/2017/UK (78)	*In-vitro* assessment of the migratory ability of Primary human umbilical vein endothelial cells in the presence of the comparative groups	AAE of Vype e-cigarettes; AAE of cigarette (3R4F)	E-cigarette AAE did not inhibit the migratory properties, even at dosage 2 times that of 3R4F. Concentration-Dependent inhibition of migration was noted for 3R4F, with a concentration of >20% eliciting complete inhibition
6	Tommasi/2017/US (79)	*In-vitro* assessment of mutagenicity on the mouse and human fibroblast cells by the comparative groups	E-cigarette extract at a concentration of 5%; e-cigarette extract at concentration of <20%	E-cigarette did not induce any significant mutagenic effect at both 5% and <20% concentration
7	Cuadra/2019/US (80)	*In-vitro* assessment of the growth of streptococci by the comparative groups	Flavorless e-cigarette aerosol (+/− nicotine); cigarette smoke	E-cigarette aerosol +/− nicotine did not have any significant effect on the microbe, while cigarette smoke caused significant inhibition
8	Thorne/2016/UK (74)	*In-vitro* assessment of the mutagenicity induced on Salmonella typhimurium by the comparative groups	ACM of e-cigarette; fresh e-cigarette aerosol; cigarette smoke (3R4F)	ACM of e-cigarette and the fresh e-cigarette aerosol did not cause any mutagenicity up to 2,400 µg/plate and 1 L/min dilution (for 3 h) respectively
9	Canistro/2017/Italy (50)	*In-vivo* assessment of the toxicity induced on rat lung model by the comparative groups	E-cigarette vapor	The significant toxic effect induced by the e-cigarette aerosol in the form of polycyclic aromatic hydrocarbons activation increased production of oxygen free radical production, DNA strand breaks, micronuclei formation, point mutations
10	Polosa/2017/Italy (59)	A clinical study assessing the long-term (3.5-year) effect of e-cigarettes on health	E-cigarette users with no history of using tobacco cigarette; never smokers	There was no significant health difference between the comparative groups
11	Manzoli/2015/Italy (60)	A clinical study assessing the 12-months effect of the comparative groups	E-cigarette users (≥50 puffs weekly) with a history of tobacco smoking; tobacco smokers (≥1 cigarette/day); dual users	Incidence of possible smoking-related diseases in the comparative groups was too scarce to comment
12	Manzoli/2016/Italy (61)	A clinical study assessing the 24-months effect of the comparative groups	E-cigarette users (≥50 puffs weekly) with a history of tobacco smoking; tobacco smokers (≥1 cigarette/day); dual users	Incidence of possible smoking-related diseases in the comparative groups was too scarce to comment
13	Flacco/2019/Italy (62)	A clinical study assessing the 4-year effect of the comparative groups	E-cigarette users (≥50 puffs weekly) with a history of tobacco smoking; tobacco smokers (≥1 cigarette/day); dual users	There was no significant difference in the incidence of possible smoking-related diseases between the comparative groups
14	Goniewicz/2018/US (68)	A clinical study comparing the tobacco-related toxicant concentration between the comparative groups	E-cigarette users; combustible tobacco cigarette users; dual users; never smokers	The highest toxicity was observed for dual users followed by combustible tobacco users, and e-cigarette users. E-cigarette users had significantly lower levels of volatile organic compounds (VOC) nicotine, metals, tobacco-specific nitrosamines, and polycyclic aromatic hydrocarbons
15	Lorkiewicz/2019/US (69)	A clinical study comparing the tobacco-related toxicant concentration between the comparative groups	E-cigarette; combustible cigarette; smokeless tobacco; never users	Compared to never users, e-cigarette users had a higher level of cyanide, xylene, styrene, benzene, and ethylbenzene. VOC, nicotine levels of E-cigarette were lower than combustible cigarette users. E-cigarette users had a higher level of xylene, N,N-dimethylformamide, and acrylonitrile than smokeless tobacco users.
16	Shahab/2017/US (70)	A clinical study comparing the tobacco-related toxicant concentration between the comparative groups	Past users of the combustible cigarette with long term (≥6 months) e-cigarette use; combustible cigarette users; nicotine replacement therapy (NRT); dual users of E-cigarettes and combustible cigarettes; dual users of combustible cigarettes and NRT	Tobacco-specific nitrosamines and VOC levels were lower in the NRT group and the e-cigarette users compared to the other comparative groups
17	Goniewicz/2016/US (52)	A clinical study comparing the tobacco-related toxicant concentration as individuals switch from combustible cigarettes to e-cigarettes	Combustible cigarette users switched to e-cigarettes	The switch did not cause change in levels of nicotine and some polycyclic aromatic hydrocarbon metabolites. The switch caused significant decrease in the levels of 1,3-butadiene, benzene, and acrylonitrile, 3-hydroxyfluorene, NNAL (a tobacco-specific nitrosamine)
18	Clemens/2019/US (81)	A clinical study comparing the tobacco-related toxicant concentration of the comparative groups	Pregnant women using an e-cigarette; pregnant women with a dual habit of e-cigarettes and combustible cigarettes; pregnant women with a habit of combustible cigarette; pregnant women who are never smokers	Tobacco-specific nitrosamines, nicotine, and cotinine levels of dual users were higher than never users and were not significantly different from combustible cigarette users
19	O' Connell/2016/The Netherlands (71)	A clinical study comparing the potentially harmful constituents in the comparative groups	Past users of combustible cigarette switched to e-cigarettes; dual users of combustible cigarettes and e-cigarettes; past users of combustible cigarette discontinued use of all tobacco-nicotine products	Potentially harmful constituents and biomarkers of exposure (BOE) were lower after switching to e-cigarettes but was higher for individuals switched to dual-use
20	D'Ruiz/2016/US (72)	A clinical study comparing the BOE in the comparative groups	Past users of combustible cigarette switched to e-cigarettes; past users of combustible cigarette discontinued use of all tobacco-nicotine products	BOE reduced for individuals shifting to e-cigarettes but increased for individuals shifting to dual-use
21	Pulvers/2018/US (73)	A clinical study comparing the tobacco-related toxicant concentration as individuals switch from combustible cigarettes to e-cigarettes	Combustible cigarette users switched to e-cigarettes	Levels of carbon monoxide, metabolites of benzene, acrylonitrile, ethylene oxide decreased in individual switching to e-cigarettes
22	Tommasi/2019/US (42)	A clinical study assessing the gene regulation in oral cells of the comparative groups	E-cigarette users; combustible cigarette users; dual users	Combustible cigarette smokers had a greater number (more than 50%) of differentially expressed transcripts than the E-cigarette group. Most aberration in cigarette smokers was in the protein-coding genes, while in the e-cigarette group most aberrations were in the regulatory non-coding RNAs. 62% and 79% of the dysregulated gene were associated with cancer in the e-cigarette and the combustible cigarette group. Wnt/Ca+ pathway was implicated as the most affected pathway in the e-cigarette group
23	Lee/2017/US (82)	*In-vivo* (mouse model) assessment of the carcinogenic potential of e-cigarette	E-cigarette smoke	Lung, heart, and bladder contained mutagens including mutagenic O6-methyldeoxyguanosines and γ-hydroxy-1, N2-propano-eoxyguanosines. The DNA-repair activity was reduced
24	Bustamante/2018/US (83)	A clinical study comparing the N'-nitrosonornicotine levels in the comparative groups	E-cigarette users; combustible cigarette users; never smokers	N'-nitrosonornicotine levels were higher in e-cigarette users than never users, but lower than combustible users
25	Ganapathy/2017/US (84)	*In-vitro* assessment of genotoxicity on oral and lung epithelial cells by e-cigarette aerosol/smoke extracts	E-cigarette aerosol; E-cigarette smoke extracts	E-cigarette aerosol caused DNA damage and suppressed the antioxidant defenses of the cells. The extract increased reactionary oxygen species and reduced total antioxidant capacity. The level of presence of 8-oxo-dG (highly mutagenic DNA lesion) was higher for the aerosol than the extract