Peer Review File

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Reviewer A

While this study's primary focus is on the role of nicotine in carcinogenesis, it also highlights how animal models may not be the best model to utilize for evaluating the role different chemicals play. Thus, in the discussion, it would be useful for the authors to comment more on the differences in the half-life of nicotine and its metabolites in mice versus humans. The fact that nicotine is more rapidly metabolized in mice/rats than in humans may further emphasize that animal models are not an accurate model to evaluate the role nicotine might play in carcinogenesis.

RESPONSE: We are in full agreement with the reviewer's comment and have added specific examples of species differences on page 17, lines 630-636, with an additional citation (Perlman, 2016) for these examples. We also incorporated the reviewer's comment in our added text. We hope that the addition of these examples to the discussion we previously provided on these mice to man topic sufficiently addresses the reviewer's concerns.

The tracked text now reads: "This poses the 'mouse to man' problem; that is, the problem of extrapolation of risk, particularly related to chemical exposure, from one species to another for many reasons, including but not limited to, size, metabolic rate, life history, diet, microbiomes, and pathogens (de Jong & Maina, 2010; Kelland, 2004; Perlman, 2016). For example, the differences in metabolic rate between mice and humans correspond to anatomic, physiologic, and biochemical differences. Therefore, in the case of this review, it is important to recognize the inherent differences that may limit the translation of animal model findings when examining the potential role of nicotine in human carcinogenesis."

Reviewer B

General comments

This is a systematic review on the possible association between nicotine per se (not accompanied by any matrix components from consumer nicotine products) and cancer observed in in vivo animal studies. The methodology applied is state of the art for the generation of systematic literature reviews. The procedures are well described and easy to follow. I have one major and a few minor points of critique, which should adequately be addressed before publication of the manuscript can be recommended.

Major point

1. As stated above, the manuscript fulfils all formal requirements of a systematic review. What

is lacking is some own input by the review authors which could guide the interested reader through the numerous studies which provide evidence for all kinds of nicotine effects. While I would totally agree with the authors' overall conclusions that 'the hetero-geneity across the studies included in this review make the interpretation and generalizability of the results difficult', I would also expect some explanation (at least a hypothesis) by the authors, why the majority of tumor progression studies show an increasing effect of nicotine. A more detailed discussion this issue would be helpful.

RESPONSE: We have addressed the reviewer's comments in added text on page 16, lines 596-605. The text reads as follows:

"Although elucidation of physiological pathways that may mediate the association between nicotine exposure and cancer initiation and progression was outside of the scope of this review, several studies included in this review proposed hypotheses that may explain this association. The proposed mechanisms vary according to the tumor model used, and include a variety of processes that are activated through activation of nicotinic acetylcholine receptors and its downstream signaling pathways, such as promotion of cell proliferation, migration, invasion, and epithelial-to-mesenchymal transition (Ben, An, et al., 2020; Ben, Sun, et al., 2020; Trevino et al., 2012; Wang et al., 2019), increased endoplasmic reticulum stress (Chien et al., 2021; Davis et al., 2009), inducing cell de-differentiation (Delitto et al., 2016), modulation of immune cell functions (Hao et al., 2013; Tyagi et al., 2021), growth factor secretion and receptor activation (Heeschen et al., 2001; Jarzynka, Guo, Bar-Joseph, Hu, & Cheng, 2006; W. Liu et al., 2015; Shimizu et al., 2019; Shin et al., 2004), increased cytokine release (Molfino et al., 2011), and suppression of apoptosis (Nakada et al., 2012)."

Minor points

2. Page 4, Line 84: Please give a reference for the PRISMA checklist already at this point in the text.

RESPONSE: The supporting reference (Liberati et al. 2009) has been added on page 5, line 126.

3. Page 4, Line 107: Please provide a reference for the PICOS approach here.

RESPONSE: Two references have been added for the PICOS approach: Booth et al., 2019 and Richardson et al., 1995 on page 6, line 151.

4. Page 6, Lines 164-165: What is a "clinical reviewer"?

RESPONSE: We have removed "clinical" on page 7, line 206.

5. Page 8, Line 255: Please explain the abbreviation "RCTs".

RESPONSE: “Randomized controlled trials (RCTs)” has been defined on page 9, line 295.

6. Page 10, Line 345: “…was no different between …”, should probably read “… was not different between …”.

RESPONSE: The text now reads “was not different” on page 11, line 384.

7. Page 11, Line 362: ‘Progression Results by Cancer Model’, I do not think that many readers are familiar with the various cancer models. A brief descript of each model would be helpful.

RESPONSE: We have shifted some of the text already provided in our first submission to provide an overview of the models at the beginning of section 3.3.2.1 (Progression Results by Cancer Model) to provide the reader with an overview of each model in addition to the potential drawbacks of each. We believe that this provides appropriate awareness to the reader before they review the presented results that follow. Page 10, line 360 now begins with the following introduction (page 11, lines 402-426; corresponding edits where text has been moved to this introduction can be found on page 12, lines 445-450, page 13 lines 473-482, and page 14, lines 491-497):

“The studies included in this systematic review included xenograft/allograft models that included allografts, human tumor cell line-derived xenografts, and patient-derived xenografts; carcinogen-induced models; and genetic models of cancer.

Inoculation of xenografts or allografts either subcutaneously or orthotopically into the tissue of interest are the most commonly used animal models of cancer, however, these methods are associated with some notable limitations and therefore, results of these studies should be interpreted with caution(Onaciu et al., 2020). A key drawback of xenograft/allograft models is that they do not recapitulate the histology of tumors, which exist as mixtures of tumor cells, neighboring healthy tissue, stromal cells, supporting vasculature, and infiltrating immune cells (Becher & Holland, 2006; Denayer, Stöhr, & Van Roy, 2014; Hemann, 2012; Onaciu et al., 2020). Carcinogen-induced tumor models are generally thought to better mimic progression of human disease compared with xenograft or allograft models (Y. Liu et al., 2015). Rodent models of chemically induced cancer have been shown to reliably mimic the mechanisms of carcinogenesis, and to resemble the clinical course of human cancers in terms of morphology, histopathology, and molecular changes. However, studies have shown that inbred strains of mice vary substantially in their susceptibility to chemically-induced neoplasia in various tissues, including lung, liver, skin, and colon (Kemp, 2015). Additionally, given that the development of cancer often results from interactions between genetic and environmental factors, recent reviews have indicated that the combined use of chemical carcinogens and genetic models of cancer is the optimal approach to unravelling human disease (Kemp, 2015). Genetic models of cancer mimic the characteristics observed in human tumors including progression from benign hyperplastic lesions into aggressive tumors and are generally preferable over xenograft or allograft models of cancer (Day, Merlino, & Van Dyke, 2015; Hollingshead, Ahalt, & Gottholm, 2012; Kemp, 2015). These models provide a means of investigating the genetic basis of cancer in immunocompetent hosts, and the interaction

between genetic and environmental factors in the development and progression of cancer. However, these models show variability in tumor latency and penetrance. Furthermore, availability of genetically engineered mice is low and their use may be costly, and thus their use is not always feasible (Day et al., 2015)."

8. Page 31, Figure 3: The legend says that it should show 'included studies by country of publication', the graph, however, is identical to Figure 2.

RESPONSE: We apologize. We see that the proof has the replicated figure but we do have the original and final figure that accurately presents publications by country. It must have been an upload error on our part. We have provided the correct attachment for Figure 3.

Reviewer C

The authors reviewed the available evidence from preclinical studies on the potential association between nicotine and the initiation and/or progression of cancer. While smoking has epidemiologically long been associated with cancer onset and poorer prognosis, the effects of nicotine on malignancy are not fully elucidated. This is of particular relevance to the tobacco smoking community and to policy makers, due to the addictive nature of nicotine, which drives smoking addiction.

The authors employ a sound design, very thorough evaluation criteria and methodology of reviewing the different articles. The PRISMA workflow diagram was used properly and the PICO respected. While the findings of all those animal studies remain inconclusive, the methodology (including the appendices) and overall layout of the manuscript could serve for future systematic reviews and are worth publishing.

Editorial remarks

1. Start with #1 in affiliations. The first author should be referred to by "1".

RESPONSE: We have corrected this on page 1, lines 1-7

2. The x-axis of Figure 3 does not match the figure caption (country).

RESPONSE: As stated in the response for reviewer B, this figure was not correctly uploaded and it is duplicative of Figure 2. We have corrected Figure 3 which now correctly identifies the axis label.

Remarks on content

1. Has any of the studies reported on plasma cotinine levels?

RESPONSE: We reported the findings from the one study that reported on plasma cotinine levels (Wong et al.; ref #76). This study's findings are presented on pages 9- 11, 13, and 15 with fully extracted data presented in Supplemental Section J.

2. Are there studies where smoking was administered to animals using systems such as the ONARES, followed by nicotine intake measurement and/or plasma cotinine levels and then effects on tumorigenesis?

RESPONSE: Thirteen studies reported biomarker of exposure data after nicotine administration, and these data are discussed in Appendix F. Only one study that evaluated tumor initiation (tumorigenesis) administered nicotine via inhalation (Waldum et al. 1996). The study reported plasma nicotine concentration over the course of the study. One other study, in which nicotine was administered orally in drinking water, evaluated tumor initiation and reported urine and plasma levels of biomarkers of nicotine exposure, including nicotine and cotinine (Murphy et al., 2011). Eleven additional studies that reported tumor progression outcomes also evaluated biomarkers of exposure. Findings of these studies are presented on pages 9-11, 13, and 15, with fully extracted data presented in Supplemental Section J.

3. It would be interesting and useful to underscore the shortcomings of animal experimentation designs (and potential solutions). Human studies are much more rigorously designed, allowing for systemic reviews and more frequent meta-analyses.

RESPONSE: We have addressed this specific and critical topic on pages 16-18 with additional text, per Reviewer A's comments.

4. Please include a paragraph or a table/diagram to highlight the areas (cancer initiation, progression, response to treatment, cancer type, etc.) where nicotine has undoubtedly proven effective. This would be the take-home message, which future research can build on.

RESPONSE: Tables 1-5 on pages 30-32 present the impact of nicotine (as indicated in the "Nicotine higher", "Effect dose-related", "Control higher", "No difference", and "Results unclear" column headings).

5. Are there plans to update your systematic review with new relevant literature (as new original articles are published)?

RESPONSE: Per the published best practices (Garner et al., 2016), several factors should be considered when deciding on updating a systematic review. The factors for consideration with this systematic review, specifically, include: whether or not there are new relevant methods, new studies, or new information (Garner et al., 2016). However, given the comprehensiveness of this review, and the preclinical nature of the current evidence base, we do not anticipate a need for an updated review until there is a sufficient number of high quality preclinical studies.