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Study Protocol

The Therapeutic Effect of Extracellular Vesicles on Asthma in Pre-Clinical Models: A Systematic Review Protocol

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Abstract: Asthma is the most common pediatric disease, characterized by chronic airway inflammation and airway hyperresponsiveness. There are several management options for asthma, but no specific treatment. Extracellular vesicles (EVs) are powerful cellular mediators of endocrine, autocrine and paracrine signalling, and can modulate biophysiological function in vitro and in vivo. A thorough investigation of therapeutic effects of EVs in asthma has not been conducted. Therefore, this systematic review is designed to synthesize recent literature on the therapeutic effects of EVs on physiological and biological outcomes of asthma in pre-clinical studies. An electronic search of Web of Science, EMBASE, MEDLINE, and Scopus will be conducted on manuscripts published in the last five years that adhere to standardized guidelines for EV research. Grey literature will also be included. Two reviewers will independently screen the selected studies for title and abstract, and full text based on the eligibility criteria. Data will be extracted, narratively synthesized and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. This systematic review will summarize the current knowledge from preclinical studies investigating the therapeutic effects of EVs on asthma. The results will delineate whether EVs can mitigate biological hallmarks of asthma, and if so, describe the underlying mechanisms involved in the process. This insight is crucial for identifying key pathways that can be targeted to alleviate the burden of asthma. The data will also reveal the origin, dosage and biophysical characteristics of beneficial EVs. Overall, our results will provide a scaffold for future intervention and translational studies on asthma treatment.

Keywords: systematic review protocol; extracellular vesicles; EVs; asthma; therapy; inflammation; respiratory disease; airway hyperresponsiveness; BALF

1. Introduction

Asthma is the most common pediatric chronic disease and affects 12.5% of children in industrialized countries [1]. Both allergic and non-allergic insults can trigger asthma symptoms, which are mainly characterized by paroxysmal episodes of breathing difficulty, chronic airway inflammation associated with narrowing of the airways, and airway hyperresponsiveness (AHR) [2–6]. Mild-to-moderate asthma is associated with airway inflammation caused by the infiltration of T-helper cell type 2 (Th2) lymphocytes, mast cells, mucus hypersecretion, and AHR [7–9]. A large majority of mild-to-moderate asthma patients have a non-eosinophilic disease phenotype [9]. Moderate-to-severe asthma involves activation of interferon (IFN)-γ-producing Th1/Th17 cells [8], monocytes [10], neutrophils, airway inflammation [7,11], elevated lung lavage and serum IgE levels [12–14], and systemic and airway accumulation of inflammatory cytokines [7,8,13,15,16]. Clinical symptoms of asthma include wheezing, shortness of breath, cough [8], tightness in the chest [17], sputum production, and a limited ability to expire air [18].

Severe asthma, to a greater extent than moderate asthma, is associated with a lower quality of life and behavioural problems in children [19]. If left untreated, asthma can lead to severe respiratory distress or even death. Medications with short and long-term action are prescribed for asthma management. These include short-acting beta2 agonists (SABA), long-acting beta2 agonists (LABA), inhaled corticosteroids [20], oral corticosteroids [21], leukotriene receptor antagonists [22], and short- or long-acting muscarinic antagonists [23]. Despite these available pharmaceutical therapies, only 50% of asthma patients meet the criteria for well-controlled asthma [24]. In fact, a section of the asthmatic population does not respond well to corticosteroids as a therapy, particularly those with a higher severity of the disease and/or those with non-eosinophilic asthma [7]. Biologic therapy offers a new therapeutic strategy for patients with severe eosinophilic asthma and/or those that do not respond to inhaled corticosteroids [25].

In recent years, the rapidly developing field of extracellular vesicles (EVs) research has provided evidence supporting the potential use of EVs as a therapy for asthma. EVs are lipid bilayer-bound vesicles released from all cells [26–28]. They function in cellular communication and transport of substances including nucleic acids, proteins, and lipids [27,28]. The three main types of EV are categorized based on their size, content and biogenesis: exosomes or small EVs (<200 nm), microvesicles or medium/large EVs (100–1000 nm), and apoptotic bodies (500–5000 nm) [27,29]. EVs can be differentiated by their isolation procedures, physical characteristics, biochemical composition, or cell origin [26,29,30]. Additionally, several methods of EV isolation are used by researchers such as size-exclusion chromatography (SEC) [31], polyethylene glycol (PEG)-based precipitation [32], and differential and density-gradient ultracentrifugation [30,33]. Each of these methods produce EVs that vary in biophysical properties such as purity, yield, stability, EV subpopulations, and functional activity [29,31–34]. Previous work in the EV field is marred with inconsistent use of names, lack of standardized techniques, isolation procedures, and poor controls, which limits interpretation of data. The Minimum Information for the Study of Extracellular Vesicles (MISEV) guidelines first established in 2014 [35], and updated in 2018 [26], are designed to ameliorate this concern by requiring a set of standardized experiments to ensure rigour and reproducibility in EV science. Therefore, it is critical that EV research and interpretation of the EV-based data is undertaken in light of the MISEV guidelines.

EVs have been extensively studied as biomarkers of various diseases, due both to the biological effects and to their inherent ease of use as a liquid biopsy. More recently, a growing body of exciting research indicates that EVs can mitigate a variety of pathological conditions such as cancer [36], cardiovascular [37], haematological [38], and respiratory diseases such as asthma [39]. EVs can modulate changes in cellular function through transference of biomolecular cargo from their intraluminal and membranal space to recipient cells. The role of EVs in the pathogenesis of asthma has been discussed in previous reviews, including a recent systematic review on the pro- and anti-inflammatory roles of EVs and their causative or protective effect on airway remodelling in asthma [4]. However, a specific investigation into the therapeutic effects of EVs, isolated according to MISEV guidelines [26], and their

role in mitigating asthma has not been conducted. We propose to address this gap in our systematic review. We will synthesize current knowledge on the therapeutic potential of EVs in alleviating asthma in preclinical research models using evidence derived from studies that meet MISEV guidelines for EV research, and that have been published in the last 5 years. The time restriction has been added to specifically select papers that have been published in accordance with the rigour and reproducibility standards as detailed in MISEV 2014 [35] and/or MISEV 2018 [26].

Objective of this Review

The objective of this systematic review is to gather, synthesize, and analyze the current research on the therapeutic effect of EVs in pre-clinical models of asthma in order to make a conclusive statement on the role of EVs as a therapeutic modality for asthma. Additionally, we hope to identify the source and biophysical characteristics of EVs that mitigate biological hallmarks associated with asthma. We will also summarize information on the target pathways involved, which will furnish readers with mechanistic insight underlying the protective effects of EVs. This will provide an overview of the recent advancements in the field, and provide guidance for further preclinical translational research in the field.

2. Experimental Section

We will conduct our methods according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA)-P protocol and Cochrane methodology. The systematic review will be registered on PROSPERO, the international prospective register of systematic reviews, *a priori*.

2.1. Inclusion Criteria

2.1.1. Population

All types of in vitro (immortalized, commercial or primary cells from people or animals with asthma or exposure to asthma-like conditions), in vivo (species including *Homo sapiens, Mus musculus, Rattus norvegicus*, and other rodent and animal models), and ex vivo models (isolated airways, lung slices, 3D-bioprinted constructs) of experimental asthma (allergic and non-allergic) or those exposed to asthma-like conditions. There is no age restriction on the study population.

2.1.2. Intervention

Treatment of recipient cells/animals using EVs and/or EV cargo content as therapy for asthma. This includes both biological EVs isolated from in vitro or in vivo preclinical models, as well as EVs isolated from living cells/animals that have been packed with biologics such as recombinant proteins, genetic material, pharmaceutical drugs.

1. Any dose of EVs;
2. Any type of EVs;
3. Any isolation method of EVs;
4. EVs isolated from any cell or animal;
5. Any number of EV treatments (single, multiple);
6. Any length of treatment (days, weeks, acute or chronic);
7. Any form of EV delivery (co-culture or incubation with cells, targeted EV delivery using ligands or genetically modified vectors, intraperitoneal (ip), intravenous (iv), intramuscular (im)or subcutaneous (sc) injections, intranasal delivery, oral gavage);
8. Any type of EV cargo (endogenous cargo such as miRNA, mRNA, protein, lipids, DNA, metabolites, or exogenous cargo where EVs were packaged with biologics including genetic material, recombinant proteins or pharmaceuticals).

2.1.3. Comparator

Healthy animal models without asthma or cells from animals or humans that have not been exposed to asthma conditions, not treated at all, conditioned media treated, sham-treated or placebo/vehicle-treated controls (e.g., phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), water, saline), studies with any comparator such as 'empty' EVs, fibroblast EVs, or EVs from a control/vehicle/placebo condition in vivo or in vitro.

2.1.4. Study Design

We will include test tube experiments in a laboratory setting, cell culture and animal research studies, cross-sectional study, and case-control study designs.

2.1.5. Outcomes

These have been chosen so that we can evaluate the biologic and physiological impact of EVs on mitigating allergic or non-allergenic asthma in vitro, ex vivo, and in vivo.

Primary Outcomes:

1. Inflammation. For in vivo models: changes in markers of inflammation including inflammatory mediators such as chemokines, cytokines, immunoglobulin E (IgE) levels, and white blood cell counts, measured using different methodologies such as Western blotting, enzyme-linked immunosorbent assays (ELISAs), mRNA, proteomics and multiplex arrays in bronchoalveolar lavage fluid (BALF). For in vitro/ex vivo models: measure markers of inflammation as described above but released by cells or ex vivo transplants in culture into the conditioned media.
2. Airway hyperresponsiveness (AHR) measured by airway resistance and elastance or indices of airway smooth muscle contraction, such as measurement of cell stiffness or deformation, or contraction of ex vivo airway tissue preparations (e.g., thin-cut lung slices, airway rings or strips).

Secondary Outcomes:

1. Serum inflammation markers (measured by cytokines, total IgE levels, activation of peripheral blood mononuclear cells [PBMCs] including dendritic cells, T and B cells, monocytes, eosinophils, neutrophils, and natural killer cells).
2. Airway remodelling as measured by: wall thickening, increased mass of airway smooth muscle, deposition and accumulation of extracellular matrix (ECM) proteins, goblet cell hyperplasia, and neo-vascularization of the airways.
3. Molecular indices of cellular signaling that are linked to pro-asthma cellular responses (contraction measured by intracellular calcium influx in cultured human airway smooth muscle cells, NfκB or STAT signalling as a index of inflammation, SMAD activation, reactive oxygen species-mediated signalling, biosynthesis of ECM proteins) in all models.

2.2. Exclusion Criteria

1. Original research published in a language other than English;
2. Not published in the last 5 years;
3. Experiments on lung disease/inflammation but not asthma;
4. Studies on therapeutic strategies for allergic asthma that do not include EVs and/or EV-cargo;
5. Studies on EVs causing asthma (pathogenic role);
6. Research on liposomes or synthetic nanoparticles;
7. Studies where EVs are not characterized according to MISEV guidelines;
8. Non-primary studies e.g., reviews and systematic reviews, editorials, and opinion articles.

2.3. Review Team Members

Two principal investigators (AS and AJH) will lead this systematic review and oversee all aspects of it including protocol development, data analysis and interpretation, manuscript preparation and submission. A health science librarian with expertise in systematic reviews (NA) will develop the search strategy and ensure it is peer-reviewed according to Peer Review of Electronic Search Strategies (PRESS) [40] as detailed below. Two reviewers (POO and JEK) will conduct the initial searches, screen and select papers that meet the criteria, extract data, and synthesize the review document with the help of an expert in conducting systematic reviews and meta-analysis (MMJ). The reviewers will screen and select the papers independently to minimize bias. Content experts will provide expertise and guidance in the following areas: asthma (AJH) and EVs (AS, TMP).

2.4. Data Sources and Search Strategy

We will search the following online databases: MEDLINE, Scopus, EMBASE, and Web of Science Core Collection to retrieve studies. The search strategies to be used for this review will be generated in collaboration with a Health Sciences Librarian (NA) with experience and expertise in designing systematic literature searches (see search strategies in Appendix A). A second information specialist with no association to the project will also review the strategy using PRESS before executing the finalized search procedure. We will include original research studies published in English from the last 5 years. This time frame was selected due to the rapid development and discoveries in the field of extracellular vesicles in recent years and the requirement of studies to abide by MISEV guidelines, first published in 2014 [35] and updated in 2018 [26]. Grey literature in the form of preprints, conference abstracts/proceedings, and patent applications will also be included from searching medRxiv, bioRxiv, and WIPO IP Portal. We will use EndNote Basic for reference management.

2.5. Study Selection Process

Title and abstract screening of the search strategy results will be done independently by the two reviewers (POO and JEK) on Rayyan [29]. The screened abstracts will be labelled based on whether they meet the inclusion criteria. Any discrepancies between the inclusion or exclusion decisions made by the two reviewers will be resolved by consensus through discussion, or adjudication by a third reviewer (AS), if necessary. Next, the two reviewers will independently screen the full texts for all citations that are marked as included. We will include the numbers of articles included at each stage of searching, screening and exclusion in a PRISMA flow diagram (see Figure 1).

2.6. Dealing with Companion and Duplicate Publications

In the event of multiple companion reports (erratum or addendums) of a study, we will use the one that has the most complete dataset relevant to our systematic review. We will enumerate the companion publications as secondary reports under the primary reference of the included study.

2.7. Data Extraction

1. A data extraction document has been created on Microsoft Excel to extract data from selected studies (see Table 1). Two reviewers (POO and JEK) will independently extract the relevant data from the included articles. The list of variables for which outcome data will be extracted is detailed in the section below. Any discrepancies between the inclusion or exclusion decisions made by the two reviewers will be resolved by consensus through discussion, or adjudication by a third reviewer (AS), if necessary.
2. Collection of study characteristics (author name, publication year, language of publication, country, study design, aim/conclusion of study, species/sex of control vs. intervention groups, sample size, outcome variables, and EV isolation, characterization, treatment methods).

3. Outcome variables will be analyzed in relation to the change in the treatment group vs. the comparator group i.e., increase, decrease or no change in cytokines in treatment vs. control group. This is being done as there are a number of different assessment methodologies that can be used to measure the outcome variable. In this case, cytokine levels can be measured by Western blotting, ELISAs, mRNA, proteomics, or multiplex arrays. Furthermore, the data will also be compounded by the inclusion of in vitro, in vivo and ex vivo studies using different preclinical models. Lastly, different inflammatory mediators can be analyzed in each study. Therefore, outcome data will be extracted as an increase, decrease or no change in the treatment group vs. the comparator control group.

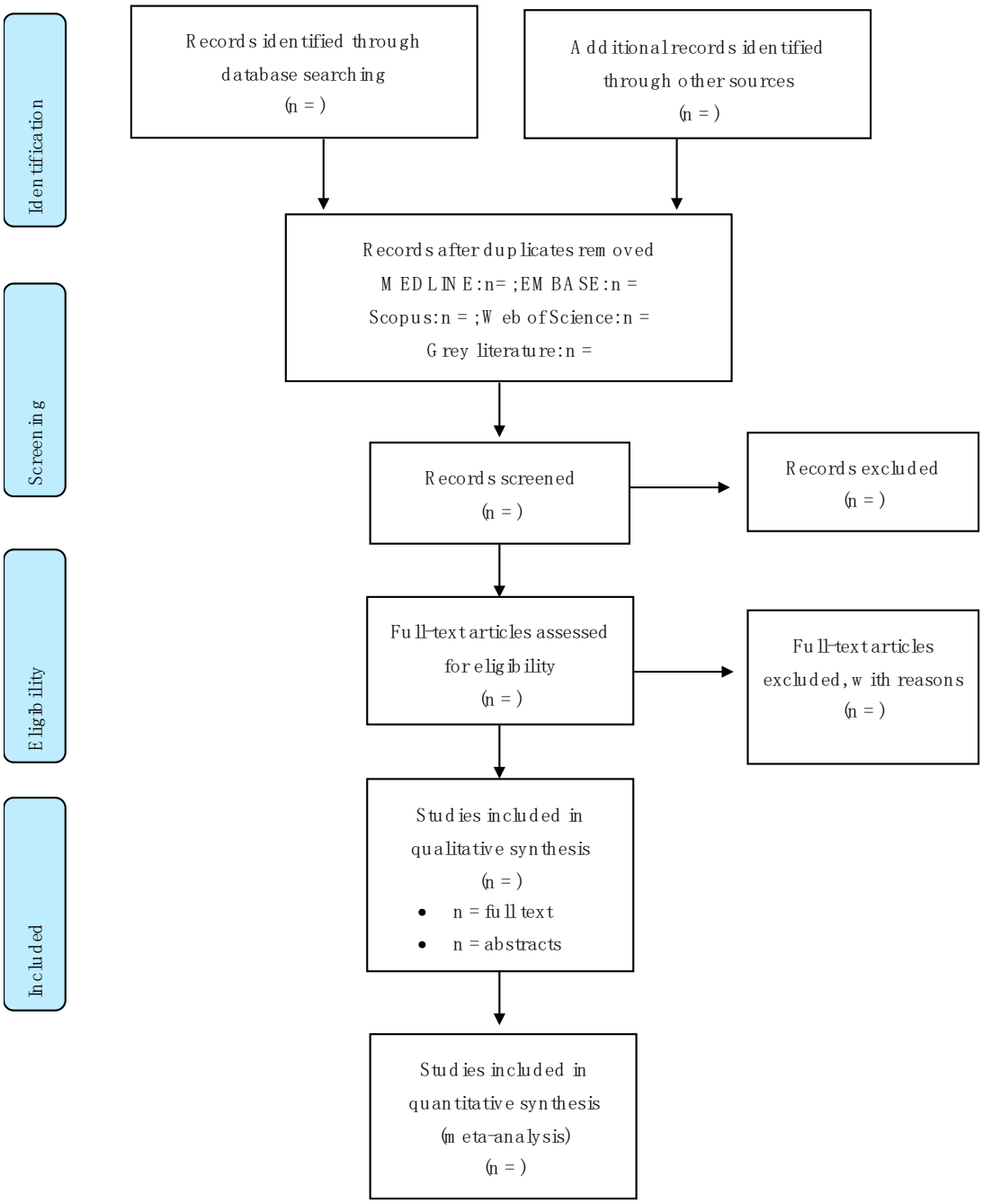

Figure 1. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram details the search and selection process that will be applied during the systematic review.

Table 1. Data will be extracted following broad categories that will include (1) overview of research, (2) summary of study design and extracellular vesicle (EV) parameters, and (3) effect of EVs on primary and secondary outcomes.

Data Extraction Variables	
Pubmed ID	Intervention: EV cargo
Authors	Intervention: EV treatment dose
Year of Publication	Intervention: EV treatment duration
Country	Intervention: EV treatment frequency
Publication Type (in vitro, in vivo, ex vivo)	Intervention: Number per independent intervention group (N)
Study Design	Intervention: Route of EV delivery (ip, iv, im, sc, co-culture)
Aim of the Study	
Conclusion of the Study	Comparator
Population: Species/type of cells	Primary Outcomes: Inflammation (in BALF and conditioned media)
Population: Sex (cells/animal)	Primary Outcomes: AHR
Population: Total number of animals used (N)	Secondary Outcomes: Serum inflammation markers
Intervention: EV type	Secondary Outcomes: Airway remodelling
Intervention: EV source	Secondary Outcomes: Molecular indices of cellular signalling
Intervention: EV isolation method	Adherence to MISEV (Y/N)

2.8. Dealing with Missing Data

We will contact study authors for missing data whenever possible.

2.9. Outcomes and Measures of Treatment Effect

AHR as measured through resistance and elastance, and inflammation in BALF (cytokines, IgE, cell counts) will be the primary outcomes of interest that will be recorded as ordinal data (increase, decrease, no change) in the intervention group vs. the comparator.

2.10. Data Synthesis

We will conduct a descriptive synthesis of the data extracted. The primary outcomes of lung function and BALF contents will be extracted from the studies as having increased, decreased, or no change. If possible, non-parametric statistical tests will be utilized to analyze the ordinal outcome measures with the help of a biostatistician.

2.11. Subgroup/Sensitivity Analysis

No *a priori* subgroup analysis is planned for the systematic review.

2.12. Risk of Bias Assessment

By use of SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE)'s risk of bias tool for animal studies and by using the US National Toxicology Program's (NTP's) Office of Health Assessment and Translation (OHAT) risk of bias rating tool for in vitro studies. We will assess a number of biases including selection bias (baseline characteristics, allocation concealment), performance bias (random housing, blinding), detection bias (blinding), attrition bias (incomplete outcome data), reporting bias (selective outcome reporting), etc. Each criterion will be assigned a value of low, high or unclear risk of bias for each included study. Risk of bias for each study will be assessed independently by two reviewers (POO and another co-author). Any discrepancies will be resolved by a senior author.

2.13. Publication Bias

We will strive to avoid publication bias by including both published and grey literature in our search. We will ensure that we extract data from studies that provide ethical approval identifying the authority that provided approval and the corresponding ethical approval code.

3. Discussion

This systematic review will synthesize information and evaluate whether EVs can be effectively used as a therapeutic strategy to control asthma in pre-clinical models. Our review is unique in that it will identify studies that strictly adhere to MISEV guidelines, and will update the previous systematic review on asthma and EVs [4]. We anticipate that this synthesis of preclinical studies will guide future research to investigate the mechanisms underlying the therapeutic effect of EVs on asthma and inform preclinical translational studies.

4. Conclusions

This systematic review will gather, synthesize, and analyze the current research on the therapeutic effect of extracellular vesicles in pre-clinical models of asthma. Our results will not only summarize the recent advancements in the field, but will also provide critical insight into the source of EVs, the EV isolation and characterization methods, dosage, mode of delivery, frequency and duration of treatment, which will provide directions for future research and help translate EV-based therapies from the laboratory to the clinic. Upon completion of our analysis, we will also be able to detail the specific mechanisms by which EVs mitigate asthma development and progression in pre-clinical models.

Author Contributions: All authors were involved in reviewing and editing the manuscript. P.O.O., J.E.K. and A.S. developed the research question, wrote and edited the systematic review protocol, and registered the protocol on PROSPERO. M.M.J. and A.J.H. provided invaluable expertise in systematic reviews and asthma research and helped develop the research question. N.A. helped to develop the search strategy and T.M.P. helped with manuscript review and editing. A.S. is the corresponding author and directly supervised the project along with A.J.H. All authors have read and agreed to the published version of this manuscript.

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Institutional Review Board Statement: Not applicable as this is a systematic review protocol.

Informed Consent Statement: Not applicable as this is a systematic review protocol.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article as this is a systematic review protocol.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A.

The search strategies that will be used in this systematic review are detailed below.

Appendix A.1. Medline Search Strategy

exp Asthma/
asthma$.mp.
(antiasthma$ or anti-asthma$).mp.
Respiratory Sounds/
wheez$.mp.
Bronchial Spasm/
bronchospas$.mp.
(bronch$ adj3 spasm$).mp.
bronchoconstrict$.mp.
exp Bronchoconstriction/
(bronch$ adj3 constrict$).mp.
Bronchial Hyperreactivity/

Respiratory Hypersensitivity/
((bronchial$ or respiratory or airway$ or lung$) adj3 (hypersensitiv$ or hyperreactiv$ or allerg$ or insufficiency)).mp.
((dust or mite$) adj3 (allerg$ or hypersensitiv$)).mp.
or/1–15
exp Extracellular vesicles/
(extracellular vesic* or extra-cellular vesic* or small vesic* or exosom* or ectosome* or nanopartic* or nano-partic* or micropartic* or micro-partic* or exovesic* or exo-vesic* or microvesic* or micro-vesic* or evs or dexosom* or apopto* bod*).mp
or/17–18
exp therapeutics/
(dt or pc or rh or th).fs
(treat* or therap* or interven*).tw,kf
or/20–22
16 and 19 and 23
limit 24 to (english language and yr = "2015 -Current")
* The asterisk serves as a wildcard symbol that broadens the search strategy by finding all the iterations of the word starting with the same letters.

The Cochrane Airway Trials Register informed the asthma portion of the search strategy.

Appendix A.2. Scopus Search Strategy

TITLE-ABS-KEY(asthma* OR antiasthma* OR anti-asthma* OR wheez* OR bronchospasm* OR (bronch* W/3 spasm*) OR bronchoconstrict* OR (bronch* W/3 constrict*) OR ((bronchial* OR respiratory OR airway* OR lung*) W/3 (hypersensitiv* OR hyperreactiv* OR allerg* OR insufficiency)) OR ((dust OR mite*) W/3 (allerg* OR hypersensitiv*)))
TITLE-ABS-KEY ((extracellular W/1 vesic*) OR (extra-cellular W/1 vesic*) OR (small W/1 vesic*) OR exosom* OR ectosome* OR nanopartic* OR nano-partic* OR micropartic* OR micro-partic* OR exovesic* OR exo-vesic* OR microvesic* OR micro-vesic* OR evs OR dexosom* OR (apopto* W/1 bod*))
TITLE-ABS-KEY(treat* OR therap* OR interven*)
#1 AND #2 AND #3
#4 AND (LIMIT-TO (LANGUAGE, "English") AND PUBYEAR > 2014
* The asterisk serves as a wildcard symbol that broadens the search strategy by finding all the iterations of the word starting with the same letters.

Appendix A.3. EMBASE Search Strategy

exp Asthma/
asthma$.mp.
(antiasthma$ or anti-asthma$).mp.
Abnormal Respiratory Sound/ or Wheezing/
wheez$.mp.
Bronchospasm/
bronchospas$.mp.
(bronch$ adj3 spasm$).mp.
bronchoconstrict$.mp.
Bronchus Hyperreactivity/
(bronch$ adj3 constrict$).mp.
Respiratory Tract Allergy/
((bronchial$ or respiratory or airway$ or lung$) adj3 (hypersensitiv$ or hyperreactiv$ or allerg$ or insufficiency)).mp.
House Dust Allergy/
((dust or mite$) adj3 (allerg$ or hypersensitiv$)).mp.
or/1–15
exosome/ or exp membrane microparticle/

(extracellular vesic* or extra-cellular vesic* or small vesic* or exosom* or ectosom* or nanopartic* or nano-partic* or micropartic* or micro-partic* or exovesic* or exo-vesic* or microvesic* or micro-vesic* or evs or dexosom* or apopto* bod*).mp
or/17-18
exp therapy/
dt.fs
(treat* or therap* or interven*).tw,kw
or/20–22
16 and 19 and 23
limit 24 to (english language and yr = "2015 -Current")
* The asterisk serves as a wildcard symbol that broadens the search strategy by finding all the iterations of the word starting with the same letters.

Appendix A.4. Web of Science Search Strategy

TS = (asthma* OR antiasthma* OR anti-asthma* OR wheez* OR bronchospasm* OR (bronch* NEAR/3 spasm*) OR bronchoconstrict* OR (bronch* NEAR/3 constrict*) OR ((bronchial* OR respiratory OR airway* OR lung*) NEAR/3 (hypersensitiv* OR hyperreactiv* OR allerg* OR insufficiency)) OR ((dust OR mite*) NEAR/3 (allerg* OR hypersensitiv*)))
TS = ((extracellular NEAR/1 vesic*) OR (extra-cellular NEAR/1 vesic*) OR (small NEAR/1 vesic*) OR exosom* OR ectosom* OR nanopartic* OR nano-partic* OR micropartic* OR micro-partic* OR exovesic* OR exo-vesic* OR microvesic* OR micro-vesic* OR evs OR dexosom* OR (apopto* NEAR/1 bod*))
TS = (treat* OR therap* OR interven*)
#1 AND #2 AND #3
(#4 AND PY = (2015-2021)) *AND* LANGUAGE: (English)
* The asterisk serves as a wildcard symbol that broadens the search strategy by finding all the iterations of the word starting with the same letters.

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