Systematic Review Protocol

The therapeutic effect of extracellular vesicles on asthma in pre-clinical models: a systematic review

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INTRODUCTION

Asthma is the most common pediatric chronic disease and affects 12.5% of children in industrialized countries¹. Both allergic and non-allergic insults can trigger asthma symptoms, which is mainly characterized by paroxysmal episodes of breathing difficulty, and chronic airway inflammation associated with narrowing of the airways, and airway hyperresponsiveness (AHR)^{2,3,4,5,6}. Mild-to-moderate asthma is associated with T-helper cell type 2 (Th2) lymphocyte activation, which involves eosinophilic airway inflammation, mucus hypersecretion, and AHR^{7,8}. Moderate-to-severe asthma involves activation of interferon (IFN)- γ -producing Th1/Th17 cells⁸, monocytes⁹, neutrophils, airway inflammation^{7,10}, elevated lung lavage and serum IgE levels^{11,12,13}, and systemic and airway accumulation of inflammatory cytokines^{7,8,12,14,15}. Clinical symptoms of asthma include wheezing, shortness of breath, cough⁸, tightness in chest¹⁶, sputum production, and limited ability to expire air¹⁷.

Severe asthma, to a greater extent than moderate asthma, is associated with a lower quality of life and behavioural problems in children¹⁸. 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 corticosteroids¹⁹, oral corticosteroids²⁰, leukotriene receptor antagonists²², and short- or long- acting muscarinic antagonists²¹. Despite these available pharmaceutical therapies, only 50% of asthma patients meet the criteria for well-controlled asthma²³. In fact, a section of the asthmatic population does not respond well to corticosteroids as a therapy, particularly in those with a higher severity of the disease⁷.

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 that are released from all cells^{24,25,26}. They function in cellular communication and transport of substances including nucleic acids, proteins, and lipids^{25,26}. The three main types of EVs 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)²⁵. EVs can be differentiated by their isolation procedures, physical characteristics, biochemical composition, or cell origin^{24,27}. Additionally, several methods of EV isolation are used by researchers such as size-exclusion chromatography (SEC)²⁸, polyethylene glycol (PEG)-based precipitation³⁰, and differential and density-gradient ultracentrifugation^{27,29}. Each of these methods produce EVs that vary in biophysical properties such as purity, yield, stability, EV subpopulations, and functional activity^{28,29,30}. 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 2014³¹ and updated in 2018²⁴ are designed to ameliorate this concern by requiring a set of standardized experiments to ensure rigour and reproducibility in EV science. EV research and interpretation of the EV-based data must be done in light of the MISEV guidelines to ensure reproducibility and rigour.

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 cancer³², cardiovascular³³, haematological³⁴, and respiratory diseases such as asthma³⁵. The role of EVs in the pathogenesis of asthma has been discussed in previous reviews, including a recent systematic review⁴ on original research conducted up to November 2018. Here, the authors evaluated both the pro- and anti-

inflammatory roles of EVs and their causative or protective effect on airway remodelling in asthma⁴. However, a specific investigation into the therapeutic effects of EVs, isolated according to MISEV guidelines²⁴, and their role in mitigating asthma has not been conducted. Therefore, the specific research question we are proposing to address is to evaluate 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 2014³¹ and/or MISEV 2018²⁴.

OBJECTIVE OF THIS REVIEW

The objective of this systematic review is to gather, synthesize, and analyze the current research on the therapeutic effect of extracellular vesicles in pre-clinical models of asthma. This will provide an overview of the recent advancements in the field and highlight potential for further research.

METHODS

We will conduct our methods according to the PRISMA-P protocol and Cochrane methodology. The systematic review will be registered on PROSPERO *a priori*.

Inclusion criteria:

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.

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.

- a) Any dose of EVs
- b) Any type of EVs
- c) Any isolation method of EVs
- d) EVs isolated from any cell or animal
- e) Any number of EV treatments (single, multiple)
- f) Any length of treatment (days, weeks, acute or chronic)
- g) Any form of EV delivery (co-culture or incubation with cells, targeted EV delivery using ligands or genetically modified vectors, intraperitoneal, intravenous or subcutaneous injections, intranasal delivery, oral gavage)
- h) 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)

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. PBS, 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*.

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.

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

Primary outcome:

- a) 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, 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.
- b) 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 outcome:

- a) 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).
- b) Airway remodelling as measured by: wall thickening, increased mass of airway smooth muscle, deposition and accumulation of ECM proteins, goblet cell hyperplasia, and neo-vascularization of the airways.
- c) 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.

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

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)³⁶ as detailed below. Two reviewers (JEK and POO) 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 on the following areas: asthma (AJH) and EVs (AS, TMPS).

Search methods for identification of studies:

We will search the following online databases: MEDLINE, Scopus, EMBASE, and Web of Science Core Collection to retrieve studies published before June 2020. 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. 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 2014³¹ and updated in 2018²⁴. 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.

Study selection process:

Title and abstract screening of the search strategy results will be done independently by the two reviewers (JEK and POO) on Rayyan³⁷. The screened abstracts will be labelled based on if 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 appendix).

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.

Data extraction:

1. A data extraction document has been created on Microsoft Excel to extract data from selected studies. Two reviewers (JEK and POO) 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.

Dealing with missing data:

We will contact study authors for missing data whenever possible.

Outcomes and measures of treatment effect:

Airway hyperresponsiveness 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.

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 changes. If possible, non-parametric statistical tests will be utilized to analyze the ordinal outcome measures with the help of a biostatistician.

Subgroup/sensitivity analysis:

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

Risk of bias assessment:

By use of SYRCLE's risk of bias tool for animal studies and by using the NTP/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 (JEK and POO). Any discrepancies will be resolved by a senior author.

Publication bias:

We will strive to avoid publication bias by including both published and grey literature in our search.

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APPENDIX

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

The search term for Scopus was:

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 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* 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 search term for EMBASE was:

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 search term for Web of Science was:

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)

PRISMA Flow Diagram:

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

Data Extraction:

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)