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Azithromycin and high-dose vitamin D for treatment and prevention of asthma-like episodes in hospitalized preschool children: study protocol for a combined double-blind randomized controlled trial

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Title

Azithromycin and high-dose vitamin D for treatment and prevention of asthma-like episodes in hospitalized preschool children: study protocol for a combined double-blind randomized controlled trial

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Tables and figures:
2 tables and 3 figures

Abbreviations:
AE = Adverse event
ALP = Alkaline Phosphatase
Ca = Calcium
COPSAC = Copenhagen Prospective Studies on Asthma in Childhood
ED = Emergency department

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EFSA = European Food Safety Authority
GCP = Good Clinical Practice
ICS = Inhaled corticosteroids
LTRA = Leukotriene receptor antagonist
OCS = Oral corticosteroids
PTH = Parathormone
Ph = Phosphorus
PCR = Polymerase chain reaction
RCT = Randomized controlled trial
RTI = Respiratory tract infection
UL = Tolerable Upper Intake Level
SD = Standard deviation
25[OH]D = 25-hydroxyvitamin D

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Abstract

Introduction

Previous randomized controlled trials (RCTs) suggest antibiotics for treating episodes of asthma-like symptoms in preschool children. Further, high-dose vitamin D supplementation has been shown to reduce the rate of asthma exacerbations among adults with asthma, while RCTs in preschool children are lacking. The aims of this combined RCT with two independent interventions, are to evaluate treatment effect of azithromycin on episode duration and the preventive effect of high-dose vitamin D supplementation on subsequent episodes of asthma-like symptoms among hospitalized preschoolers.

Methods and analysis

Eligible participants are 1–5-year-old children with a history of recurrent asthma-like symptoms, hospitalized due to an acute episode. Participants will be randomly allocated 1:1 to azithromycin (10 mg/kg/day) or placebo for 3 days (n=250). Further, independent of the azithromycin intervention, participants will be randomly allocated 1:1 to high-dose vitamin D (2000 IE/day) or placebo for 1 year (n=320). Participants are monitored with electronic diaries for asthma-like symptoms, asthma medication use, adverse events, and sick-leave from day-care. The primary outcome for the azithromycin intervention is duration of asthma-like symptoms after treatment. Secondary outcomes include duration of hospitalization and anti-asthmatic treatment. The primary outcome for the vitamin D intervention is the number of exacerbations during the treatment period. Secondary outcomes include time to first exacerbation, daily symptom burden, asthma medication need and safety.

Ethics and dissemination

The study is approved by the Danish local ethical committee (H-20065249, H-20066827) and is reviewed by Medicines Authority. The study is monitored by the local Unit for Good Clinical Practice and is conducted in accordance with the guiding principles of the Declaration of Helsinki. The combined RCT with the two independent interventions are registered at https://eudract.ema.eurupa.eu and clinicaltrials.gov (in progress). The results will be published in peer-reviewed journals and presented at international conferences.

Trial registration number

https://eudract.ema.eurupa.eu, 2020-004420-42, 2020-004421-23.

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Strengths and limitations of the study
• (+) This is a large combined RCT consisting of two independent interventions, which evaluate if a 3-days course of azithromycin (10 mg/kg/day) compared to placebo reduces the duration of asthma-like episodes in hospitalized preschool children, and if high-dose vitamin D supplementation (2000 IE/day) for 1 year compared to placebo reduces the subsequent number of asthma-like episodes requiring oral corticosteroids and/or emergency department visits and/or hospitalization.
• (+) If the interventions are proven effective, this RCT could have a huge impact on pediatric asthma management as the findings can be directly implemented in clinical practice and thereby address an unmet clinical need for treating and preventing recurrent asthma-like episodes in young children.
• (+) The children are carefully monitored with electronic diary recordings of asthma-like symptoms, anti-asthmatic treatment, and adverse events assuring robust and clinically relevant efficacy and safety endpoints.
• (+) Exploratory effect modifiers in both study arms include the airway immune profile, respiratory pathogens, airway microbiome assessments, blood eosinophil count, total-IgE and specific IgE levels and genetic markers, which enable analyzing underlying mechanisms of treatment effects and whether there are specific phenotypes or episode characteristics, where azithromycin and vitamin D treatment are more effective.
• (-) The azithromycin intervention could raise concern regarding antibiotic resistance and microbial derangements.

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INTRODUCTION

Childhood asthma is often preceded by recurrent episodes of asthma-like symptoms during the first years of life [1]. Severe episodes with asthma-like symptoms cause a high rate of emergency department (ED) visits and often require hospitalization [2,3] with high socioeconomic demands [4]. Typical triggers for asthma-like symptoms are respiratory tract infections (RTIs) of both viral and bacterial etiology [5,6], exercise, allergens, pollutants, and tobacco smoke. Exacerbations in preschoolers are most often triggered by RTIs [7] and have a poor response to oral corticosteroids (OCS) that are one of few options for treating exacerbations in this age group [8]. Preventive treatment of asthma in preschoolers is maintenance inhaled corticosteroids (ICS) as monotherapy and/or an oral leukotriene receptor antagonist (LTRA), which is effective for achieving daily symptom control, whereas many children still experience exacerbations requiring OCS and/or hospitalization [9]. Currently, there are no interventions aiming at reducing the high morbidity associated with acute exacerbations, which is a major unmet clinical need in this age group of vulnerable young children[10].

Azithromycin for treatment of asthma-like episodes

Antibiotics are not recommended for treating exacerbations of asthma-like symptoms in young children, although they are commonly used for this purpose [6,11]. As exacerbations of asthma-like symptoms can be triggered by bacterial RTIs, we previously conducted a randomized controlled trial (RCT) of azithromycin vs. placebo including 158 episodes of asthma-like symptoms in 72 children from the $COPSAC_{2010}$ cohort aged 12-36 months with a history of recurrent asthma-like symptoms [12]. The RTC showed that 3-days azithromycin (10 mg/kg/day) reduced the episode duration by 63% compared to placebo. Furthermore, the airway microbiota and airway immune responses during the episode was shown to predict children, who benefitted from the treatment [13,14].

A comparable American RCT included 443 children aged 12-71 months with a history of severe episodes with asthma-like symptoms showing that 5-days azithromycin (12 mg/kg/day) reduced the risk of progression from mild to severe lower respiratory tract illness with need for OCS by 36%, but episode duration was not reported [15]. A Canadian RCT included 222 children aged 12-60 months from an ED, admitted with acute asthma-like symptoms showing no effect on the duration of symptoms from 5-days azithromycin course (10 mg/kg/day) [16]. This study differed from the American RCT and our previous RCT as the children did not have a history of recurrent asthma-like symptoms. However, a sub-analysis among children with recurrent asthma-like symptoms showed similar results [16], but had a low number of subjects in the subgroup.

High-dose vitamin D for prevention of exacerbations

In children with asthma, vitamin D insufficiency is prevalent year-round and may increase the risk of exacerbations [17,18]. A recent systematic review and meta-analysis, which included several RCTs of vitamin D supplementation in children and adults showed that vitamin D significantly reduced exacerbations requiring OCS by 36% (n=658 adults; 22 preschoolers) and ED visits or hospitalizations by 61% (664 adults; 277 school-aged children; 22 preschoolers) compared to placebo [19]. The systematic review underlines the shortage of RCTs in children, especially in preschoolers, where the largest preventive effect is to be expected as vitamin D has been shown to reduce the risk of RTIs [20], which are the main trigger of exacerbations in young children.

Currently, there are 7 published pediatric vitamin D RCTs with respiratory outcomes [21], but only one is among preschoolers, which is a safety pilot study of the DIVA RCT (NCT03365687) randomizing 22 preschool-aged children to an oral bolus of 100.000 IU plus 400 IU/day for 6 months or placebo. This was safe and raised serum 25-hydroxyvitamin D (25[OH]D) ≥75 nmol/L, but was underpowered to show differences in unplanned healthcare attendances due to acute wheeze [22]. Apart from DIVA there are no other registered RCTs of vitamin D supplementation to preschoolers

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with asthma-like symptoms. Therefore, RCTs in preschoolers are needed to establish whether high-dose vitamin D supplementation can prevent exacerbations with asthma-like symptoms requiring OCS and/or ED visits and/or hospitalization [23].

It is important to investigate potential mechanisms of both azithromycin and high-dose vitamin D to be able to pinpoint specific phenotypes and episode characteristics, where treatment may be most effective.

Objectives

The main objectives are to evaluate if 3-days azithromycin treatment (10 mg/kg/day) reduces the current episode duration, and if 1-year vitamin D supplementation (2000 IE/day) reduces the number of subsequent episodes requiring OCS and/or ED visits and/or hospitalization. This will be studied among hospitalized children aged 1-5 years with a history of recurrent asthma-like symptoms.

METHODS

This study is a combined double-blind RCT with two independent interventions. Children, who meet the inclusion criteria for both interventions, will be asked to participate in both - one treatment arm consisting of azithromycin versus placebo and a second arm of vitamin D versus placebo (see figure 1). The study is coordinated by researchers at the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) research unit at Herlev-Gentofte Hospital.

Participants, intervention, and outcomes

Study setting

The study will be conducted at four pediatric departments in Zealand, Denmark: Herlev-Gentofte University Hospital, Hvidovre University Hospital, Nordsjællands University Hospital and Slagelse Hospital. Additional pediatric departments will be included if needed.

Eligibility criteria: inclusion/exclusion

The inclusion criteria for participation in both study arms are: (i) hospitalization due to an episode of asthma-like symptoms, (ii) age 12-71 months, (iii) a medical history of episode(s) with asthma-like symptoms and treatment with SABA as monotherapy, or in combination with ICS and/or LTRA. Further, parents must have fluent Danish skills. The specific inclusion and exclusion criteria for participation in the two study arms are listed in Table 1.

The exclusion criteria aim to minimize the risk of allergic reactions and of including children, who suffer from pneumonia or other bacterial infections, rickets, severe vitamin D deficiency, and risk of vitamin D intoxication.

Interventions

In the azithromycin arm the children are randomized to a 3-days course of azithromycin (10 mg/kg/day) or placebo. The interventions consist of powder for oral suspension 40 mg/mL azithromycin or placebo in identical looking 22,5 mL packings. The parents will report their child's asthma-like symptoms, use of asthma medication, adverse events (AEs), and sick leave via a link sent per text messaging once daily for 21 days via the secure system Research Electronic Data Capture (REDCap).

In the vitamin D arm, the children are randomized to one daily dose of an oral suspension of 2000 IE (~50 µg) vitamin D or an identical volume of lookalike placebo for 1 year. A total intake of 2400 IU/day (~60 µg/day) is accepted as children aged 14 days to 4 years and from 4 years in the winter months are recommended by the Danish Health Authority to take a supplement of 400 IE/day (~10

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μg) [24]. To avoid seasonal variations in 25[OH]D levels at baseline and the seasonal variation in RTI, the intervention is given for 12 months. The parents will report their child's asthma-like symptoms, use of asthma medication, AEs, and sick leave via a weekly REDCap link, where they can fill out the daily information from the prior week (See Figure 2).

Outcomes

The primary outcome in the azithromycin study arm is the duration of the episode with asthma-like symptoms after randomization. Secondary outcomes are: (i) symptom burden; (ii) length of hospitalization; (iii) intensity of episode treatment; (iv) leave from daycare; (v) health economic benefits; (vi) gut microbiome and resistance profiles. Evaluation of effect modification by (vii) airway microbiome (virus detection by polymerase chain reaction (PCR), bacterial culturing and 16s sequencing), and (viii) airway immune profile. Further, safety by (ix) AEs for three weeks.
The primary outcome in the vitamin D study arm is the number of episodes with asthma-like symptoms requiring OCS and/or ED visit and/or hospitalization over a 12-month period after randomization. Secondary outcomes are: (i) time to first exacerbation; (ii) number of episodes with asthma-like symptoms; (iii) length of hospitalization; (iv) symptom burden between exacerbations; (v) treatment during exacerbations; (vi) step-down asthma maintenance therapy; (vii) health economic benefits. Evaluation of effect modification by (viii) baseline 25[OH]D level; (ix) genetic variation and expression in the vitamin D pathway, *e.g.,* VDR and VDBP; (x) genetic variation and expression in known childhood asthma loci, *e.g.,* 17q21; (xi) airway microbiome; (xii) airway immune profile and (xiii) atopic status, *i.e.,* blood eosinophil count, total-IgE and specific-IgE levels towards aeroallergens. Further, safety profile by clinically significant; (xiv) hypercalciuria; (xv) hypercalcaemia; (xvi) 25[OH]D>250 nmol/L, and (xv) AEs for one year. Furthermore, (xvi) prevention of and possible reduction of symptom burden from COVID-19.

Participant timeline

Recruitment of participants will start fall 2021 and is expected to last 3 years. The study will be completed when at least 250 participants are recruited for the azithromycin study arm and 320 participants for the D vitamin study arm. Study duration per participant for the azithromycin arm is 21 days, the first 3 days with treatment, and fecal samples are further collected at inclusion, 21 days, 3 months and 1 year after intervention. Study duration per participant for the vitamin D arm is 1 year (See Figure 3). The estimated completion of the study is fall 2025.

Recruitment

A doctor and a nurse from the research team will contact the pediatric wards every morning on weekdays to screen for admitted patients eligible to include in the study. If this is the case, layperson summaries, including participant information and the informed consent form, which describes the experiment, and its procedures will be handed to the parents. If the parents after reading the layperson summaries are interested in participating, the research team will make an appointment with the family on that day or within 3 days, while the child is still hospitalized to inform orally about the studies purposes, content, risks, form etc. Here, the parents will have the possibility to ask questions before finally deciding to participate.

Adverse events

Azithromycin is a commonly used macrolide in children and is shown safe in several RCTs including the RCT performed in the $COPSAC_{2010}$ cohort [12,25,26]. Of known AEs the most frequent are gastrointestinal (*i.e.,* diarrhea, nausea, vomiting), whereas other AEs are rare (*e.g.,* allergic reaction).

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AEs due to vitamin D treatment are rare. The European Food Safety Authority (EFSA) has provided Tolerable Upper Intake Levels (ULs) of vitamin D for children of all ages, concluding that for 1 to 10-year-olds, UL is 2000 IU/day (~50 μg) [21]. Recently, the TARGet Kids! Collaboration conducted a RCT randomizing 703 healthy 1-5-year-old children to 2000 IU/day or 400 IU/day for a minimum of 4 months to reduce RTIs, where no vitamin D related AEs were reported [27]. Based on above mentioned studies the intervention dose chosen in this RCT is 2000 IU/day. Potential AEs of high-dose vitamin D treatment include (i) hypocalcemia; and (ii) hypercalcaemia/calciuria. These AEs are rare and are assessed at inclusion, after 3 months, and at a 1-year follow-up visit by measuring 25[OH]D, Calcium (Ca), Phosphorus (Ph), Alkaline Phosphatase (ALP), Parathormone (PTH) and urine Calcium:Creatinine ratio, which will be evaluated by an independent pediatrician.
If a child unexpectedly develops symptoms of severe AEs, such as intoxication or allergic reaction, treatment will be stopped immediately, randomization unblinded, and the participant will be referred for assessment and treatment.

Sample size

The main outcome in the azithromycin study arm is the duration of the episode with asthma-like symptoms in days; *i.e.* non-normally distributed. Due to some observations of zero days duration, we will add a pseudocount of 1-day duration for transformation of all durations: $\log(\text{duration}+1) = \beta_0 + R*\beta_1 + \text{error}$, where R is the intervention. Based on the $COPSAC_{2010}$ RCT duration of symptoms [12], we expect a standard deviation (SD) of log(duration +1) = 1.04. With a power of 80% and a two-tailed alpha (α) of 5%, a delta (λ) in geometric mean ratio of 1.5, corresponding to a 50% reduction in symptoms duration, we need a sample size of n = 230 children randomized in two groups on a 1:1 and expecting a drop out of 10%. To increase power, we aim to recruit 250 children (125/arm).
The main outcome in the vitamin D study arm is the number of episodes with asthma-like symptoms requiring OCS and/or ED visit and/or hospitalization over a 12 months period (λ), which is estimated at 0.94/child in the placebo arm based on previous RCTs with ICS [28]. A sample size of 145 children per arm will provide 80% power with a two-tailed α of 5% to detect a 35% relative reduction in the mean number of events (*i.e.*, λ=0.94 in the placebo vs. λ=0.611 in the intervention group), based on a previous observed rate ratio of 0.64 for exacerbations requiring OCS in a meta-analysis [19]. We expect a 10% drop out and therefore aim to recruit 320 children (160/arm).

Blinding and randomization

The randomization in both study arms will be done at the Capital Region Pharmacy by a computer-generated list of random numbers in blocks of 10 and copies of the randomization code will be kept in sealed envelopes at the research site and at the Capital Region Pharmacy.
Treatment compliance in the azithromycin study arm is ascertained by parents checking a REDCap link checkbox after administration during the first three days.
Treatment compliance in the vitamin D study arm is ascertained by weighting of returned bottles at the 1-year follow-up visit. All participants, parents, and members of the research team are blinded to group allocation. Efficacy and safety analyses will be performed under allocation concealment. Both study arms will remain double-blinded until all children have been recruited and throughout the data validation and primary outcome analysis phases.

Data collection, management, and analyses

Data collection methods

Baseline health, environment and sociodemographic data from the child and parent(s) will be collected through an interview and directly entered in the REDCap trial database. Moreover, we will extract data on prescribed medication and information from the child's medical records from birth

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and 4 years ahead from date of inclusion. From the diary data registered online via the REDCap system, we will monitor asthma-like symptoms as in our previous azithromycin RCT [12,29]. The parents will report daily for 21 days their child's asthma-like symptoms, use of medication, possible AEs, and sick leave. A link for this registration is sent once daily via REDCap. If the child also participates in the vitamin D study arm, the parents continue to fill out the diary once weekly for 1 year.

The participants included in the vitamin D study arm will after 3 months attend their local hospital for a blood sample and urine sample, measuring Ca, Ph, ALP, PTH and urine Calcium:Creatinine ratio, which will be evaluated by an independent pediatrician for signs of vitamin D intoxication. Further, they will attend a clinical follow-up visit at the COPSAC research clinic 1 year after enrolment for collection of a variety of bio samples, screening for AEs, and interviewing the parent(s) regarding the recent years' exacerbations, medication use, and step-up/step-down in preventive asthma medication.

The planned study procedures are listed in Table 2.

Statistical methods

An intention-to-treat analysis will be carried out with all randomized children by treatment group.

Azithromycin study

We will analyze the duration of an episode of asthma-like symptoms and SABA use after treatment with Quasi-Poisson regression models. This type of regression is ideal for modelling counts, such as duration because it captures both skewness and variance heterogeneity and provides an easy-to-interpret quantification of effects as relative change in mean counts. We will evaluate potential effects of factors such as recruiting center and age of the child by inclusion of these in the models. We will analyze baseline factors potentially modifying the treatment effect such as airway immune profile, airway microbial colonization, and clinical appearance including current use of anti-asthmatic treatment as interaction terms in the models. This is planned as we previously showed that lower levels of TNF-alpha and CCL22 and higher IL-10 in the upper airway predicted better treatment response to azithromycin [14]. Furthermore, we showed that the composition of the airway microbiota during an acute episode modified the effect of azithromycin treatment with high richness or high relative abundance of *Veillonella* and *Neisseria* being associated with a better treatment effect of azithromycin [13]. Safety analyses will include all children who receive the intervention, including those, if any, without a primary outcome measure.

Vitamin D study

We will analyze the number of exacerbations requiring OCS and/or ED visit and/or hospitalization per child during the 12-month follow-up using a Quasi-Poisson regression model to compute the incidence risk ratio.

Six subgroup analyses are planned: 25[OH]D (<75 vs ≥75 nmol/L), sex (male/female), atopy (sIgE ≤0.35 or >0.35 kUa/L), elevated blood eosinophil count (≤0.5 or >0.5 x10^9cells/mL), BMI (Z-score ≤2 vs >2 SD), and ethnicity (Caucasian vs non-Caucasian).

Effect modification analyses are planned for baseline airway immune mediator levels, respiratory pathogens (virus and bacteria), airway microbiome, and for genetic variations in the vitamin D pathway/metabolism and childhood asthma risk loci.

The time to first exacerbation requiring OCS and/or ED visit and/or hospitalization will be compared between groups using a cox regression model. Symptom/hospitalization duration during an exacerbation will be compared between groups using a Quasi-Poisson or general linear regression

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model. Similar analyses will be carried out for intensity of SABA and OCS use. Daily symptom burden between exacerbations will also be compared using a Poisson or general linear regression model. The Chi-square test or Mantel-Haenszel method will serve to compare categorical outcomes, including the number of children with ≥1 episode of clinically significant hypercalciuria, hypercalcemia, elevated serum 25[OH]D, and AEs.

No adjustment for multiple outcomes is planned in any of the study arms.

Data management and monitoring

Acquired data is stored in the secure REDCap system provided by the Capital Region of Copenhagen. The data are directly entered electronically, both by researchers and the parents. Both study arms will be monitored by the unit of Good Clinical Practice (GCP), Bispebjerg Hospital. GCP will oversee randomization, data management, progress monitoring and all analyses. A combination of remote monitoring activities and routine monitoring visits are conducted to ensure that each site adheres to the study protocol, GCP guidelines and data collection completeness.

Ethics and dissemination

The studies are conducted in accordance with the guiding principles of the Declaration of Helsinki and are approved by the Danish ethical committee and are reviewed the Danish Medicines Agency. All families will receive written information about the study prior to participation and both parents will give verbal and written informed consent before enrolment. Acquired data is stored in the secure REDCap system. No provision is given to the participants, and they have the possibility to withdraw from the study at any time.

No participant identifiers will be used in the dissemination of this research. Results will be disseminated to the medical community via national/international conferences and publications in peer-reviewed journals, to lay people via social and public media, and to families of preschoolers with asthma by involving patient organizations and networks. All results, negative as well as positive, will be published. Further, the results will be available on our websites: www.copsac.com and www.dbac.dk. The studies are registered at https://eudract.ema.eurupa.eu and https://clinicaltrials.gov (in progress).

DISCUSSION

This paper describes the design and methodology of a combined RCT, consisting of two independent interventions with the overall aim to improve both secondary prevention (high-dose vitamin D) and tertiary prevention (azithromycin) of asthma-like symptoms in preschoolers. This has the potential to fill in a huge medical need as the prevalence of childhood asthma has increased markedly in the last decades [2] and there are limited treatment possibilities for both secondary and tertiary prevention of exacerbations.

We hypothesize that azithromycin treatment shortens episode duration of asthma-like symptoms in hospitalized preschool children. If successful, the project will provide evidence of azithromycin as a potential standard treatment for recurrent acute asthma-like episodes requiring hospitalization. A secondary aim is to assess the individual responses to azithromycin treatment based on the airway immune profile and the airway microbiome. Previously, we investigated levels of 18 cytokines and chemokines *in vivo* in the upper airway to assess the value of these mediators for predicting treatment response to azithromycin. Levels of TNF-α, IL-10 and CCL22 were found to predict treatment response [14]. Further, in the same study, we also found that the composition of the airway microbiota during the acute episode modified the treatment effect of azithromycin. A high

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bacterial richness was associated with a better treatment effect and high relative abundance of several individual bacterial taxa similarly increased the treatment effects [13]. If replicated, these results may pave a path for a personalized treatment strategy from immunological and/or microbial profiles, so only children, who will benefit from azithromycin will be treated.

The azithromycin study can raise concerns regarding antibiotic resistance. However, the intervention only targets children with recurrent asthma-like episodes, who currently receive or have received anti-asthmatic medication, which narrows the group, where the treatment will be relevant. A focus on personalized treatment in the future will further narrow down the group, where azithromycin is indicated. Finally, we have previously reported that azithromycin treatment only leads to short-term deranged gut microbiota (*i.e.,* 21 days after intervention), which was normalized later in childhood (*i.e.,* 1-3 years after intervention) [30].

We hypothesize that high-dose vitamin D treatment will lead to more effective secondary prevention of episodes with asthma-like symptoms in preschoolers currently receiving or having received anti-asthmatic medication. We will investigate potential mechanisms of action to elucidate the protective role of vitamin D, which includes effect modification analyses of airway immune mediator levels, the airway microbiome, and genetic variations and gene expression in the vitamin D pathway/metabolism and known childhood asthma risk loci. This strategy has the potential to highlight specific phenotypes of asthma and episode characteristics, where vitamin D supplementation is most effective. Importantly, vitamin D supplementation has been found to reduce the risk of RTI in a subgroup analysis within a meta-analysis of children aged 1–16 years by 40% (n = 513 children) [20]. This suggests that vitamin D may be particularly beneficial for prevention of episodes in preschoolers, where RTI is the main trigger of exacerbations. This effect on RTIs further suggests that vitamin D has its impact via the immune system. Hence, exploring the role of baseline vitamin D levels and the impact of vitamin D on airway immune mediators would enhance our understanding of mechanisms of action.

This combined placebo-controlled, double-blind, RCT with two independent interventions has several strengths, which include (i) enrolment of hospitalized preschoolers with asthma-like symptoms irrespective of atopy, phenotype and baseline 25[OH]D (vitamin D arm); (ii) a short-term (*i.e.* 3 days for azithromycin), safe, and easy single daily dosing without bolus treatment for vitamin D arm; (iii) a 12-months intervention for vitamin D ensuring that the study period continues throughout winter and covers the peak incidence of exacerbations and RTIs and the seasonal decline in 25[OH]D; (iv) primary outcomes that address unmet clinical and societal needs; (v) exploration of potential mechanisms of action to direct personalized treatment; (vi) safety profile documentation; and (vii) interventions that can easily be implemented in clinical practice. RCTs are necessary as a basis for evidence-based guidelines before being able to (i) implement azithromycin as part of the tertiary prevention of asthma in preschool children and (ii) implement vitamin D supplementation as a part of secondary prevention of asthma in preschool children.

The project will be performed by the research team at COPSAC in collaboration with pediatric dept. at Herlev-Gentofte University Hospital, Hvidovre University Hospital, Nordsjællands University Hospital and Slagelse Hospital, Denmark.

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We used the SPIRIT checklist when writing this report.

Contributors

BC, JS, HB, KB and JNK designed the studies. JNK was responsible for writing the first draft of the manuscript. UR were responsible for the data system REDCap and contact to the pharmacy and the GCP unit. JNK, BC, JS, UR, KB, HB read and critically revised the manuscript.

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Competing interests

None declared.

Patient consent for publication

Not required.

Public and Patient Involvement

No patients were involved in setting the research question or the outcome measure, nor are they involved in developing plans for recruitment, design, or implementation of the study. No patients will be asked to advise on interpretation or writing up of results. We will disseminate the results of the research to study participants and public.

Provance and peer review

Not commissioned, externally peer reviewed.

Open access

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Data sharing statement and ethical approval

The studies will record two datasets on in total 250 participants in the azithromycin randomized controlled trial (RCT) and 360 participants the vitamin D RCT, most likely participants will be included in both studies. We collect from interviews and medical records demographic-, growth-, birth data, data on atopic status, medication, and former episode(s) with asthma-like symptoms. Further, we collect bio samples, where data from laboratory analysis will be stored. Researchers are all blinded while the trials include and follow the participants. The data are stored in the secure system Research Electronic Data Capture. The studies are approved by the local ethical committee and reviewed by Danish Medicines Authority. Further, the studies are monitored by the local Unit for Good Clinical Practice. Data will be stored for 10 years from collection date and hereafter it will be destroyed. The data from clinical trials to be made available upon reasonable request.

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Tables

Table 1 *Overview of the Inclusion and exclusion criteria for azithromycin and vitamin D.*

Inclusion Criteria	
Azithromycin and vitamin D study	• Hospitalization due to an episode of asthma-like symptoms • Age 12-71 months • Medical history of episode(s) with asthma-like symptoms and treatment with SABA as monotherapy, or in combination with ICS and/or LTRA
Exclusion Criteria	
Azithromycin and vitamin D study	• Clinical signs of pneumonia (tachypnoea: Respiratory rate >50 and/or fever: temperature >39°C and/or C-reactive protein >50) • Chronic lung disease (other than asthma/wheeze) • Impaired liver or kidney function • Neurological or psychiatric disorders • Congenital or acquired prolonged QT interval • Clinically relevant bradycardia, cardiac arrhythmia or severe heart failure.
Azithromycin study	• Macrolide allergy
Vitamin D study	• Daily intake of vitamin D supplementation or receives a combination of vitamin and dietary supplements containing vitamin D > 400 IU/day (~10 µg/day)* • Malnourished (i.e. children >2 years - age-specific BMI less than the 3rd percentile and children <2 years - weight or height in relation to age are less than the 3rd percentile) • Newly arrived refugee/immigrant from regions with high incidence of rickets • Receives medication that alters calcium or Vitamin D absorption/metabolism • Allergy to peanuts (the carrier in vitamin D drops and placebo is peanut oil)

** 2400 IU/day (~60 µg/day) is accepted for children included, as children from 14 days to 4 years and from 4 years in the winter months (October to April) are recommended by the Danish Health Authority to take a D vitamin supplement of 400 IU/day.*

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Table 2 *Overview of data we will access at the time of inclusion and over study procedures. If the child is participating in both study arms the overlapping procedures will only be taken once.*

Participant	Baseline	21 days	3 months	1 year	Description		References
Azithromycin study arm							
Fecal sample	X	X	X	X	Measurement of gut microbiota using 16S rRNA sequencing		[30]
Diary	X	X			Diary on asthma-like symptoms, medication, sick leave, and AEs is registered daily by parents via text messaging through REDCap		[12,29]
Hypopharyn-geal aspirate	X				Measurement of airway microbiota, pathogenic bacteria and vira		[31]
Filter-paper sampling	X				Evaluation of immune mediator profiles in the upper airway epithelial lining fluid		[32]
Blood sample	X				Inflammatory markers (i.e., CRP, white blood cell count)		
Anthropo-metrics	X				Height, weight		
Physical examination	X				Assessment of fever, tachypnoea, chest recessions, wheezing, and lung and heart auscultation, and examination of the skin, ears, nose, and throat		
Interview	X				Baseline characteristic	Age, sex, ethnicity, address	
					Growth data	Weight, height, BMI z-score	
					Birth data	Gestational age, birth weight and length, delivery mode, siblings at birth.	
					Atopic status	Food allergies, allergic rhinoconjunctivitis, atopic dermatitis	
					Asthma medication	SABA, ICS, OCS, LTRA (current and former)	
					Former episode(s) with asthma-like symptoms	Time, numbers, treatment (incl. medication and ER visit(s), hospitalization(s), visit to general practitioner or treated at home)	
					Other relevant data	Daycare status (if yes - also start date), smoking in the home, animal in the home	
					Parents and sibling(s) atopic status	Asthma diagnosis, food allergies, allergic rhinoconjuctivitis, atopic dermatitis	
					Socioeconomic status	Parent's education level, parents age, parents' income	
Vitamin D study arm							
Diary	X			X	Diary on asthma-like symptoms, medication, sick leave, and AEs is registered daily the first 21 days, afterwards weekly, by the parents, through text messaging via REDCap		[12,29]
Hypopharyn-geal aspirate	X			X	*		[31]
Filter-paper sampling	X			X	*		[32]
Nasal scrape	X			X	Gene expression		[33]
Urine sample	X		X	X	Calcium:Creatinine-ratio		
Anthropo-metrics	X				*		
Physical examination	X				*		
Blood sample	X		X	X	At inclusion and at the 1-year follow-up visit: 25OHD, Ca, Ph, ALP, PTH, CRP, white blood cell count. Gene expression and methylation in vitamin D pathways and childhood asthma risk loci. At 3 months after inclusion 25OHD, Ca, Ph, ALP, PTH. Total-IgE and specific-IgE towards inhalant allergens will be examined after 1 year.		
Interview regarding exhibitions	X				*		

**As in the Azithromycin study arm*

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Figure legend/caption

Figure 1 *Flow diagram of enrolment of patients. Patients who meet the inclusion criteria in both study arms (i.e vitamin D and azithromycin) can participate in both studies independently of each other.*

Figure 2 *Diary the parents fill out on information on asthma-like symptoms, asthma medication use, AEs, and sick leave. Further, a checkbox for administration of azithromycin is added to diary during the first three days.*

Figure 3 *Overview of the study timeline for both study arms (i.e., 21 days in the azithromycin arm and 1 year in the vitamin D arm).*

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	ItemNo	Description	Page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	P. 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	P. 3, P. 10
	2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	3	Date and version identifier	Headnote at all pages.
Funding	4	Sources and types of financial, material, and other support	P. 12
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	P. 1, P. 12
	5b	Name and contact information for the trial sponsor	P. 1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	P. 12
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	P. 12
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	P. 5-6, P. 4 (box regarding strength and limitation)
	6b	Explanation for choice of comparators	P. 5-6
Objectives	7	Specific objectives or hypotheses	P. 6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	P. 6

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Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	P. 6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	P. 6, P. 19 (Table 1)
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	P. 6-7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	P. 8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	P. 8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	P. 6, P. 19 (Table 1)
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	P. 7
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	P. 7
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	P. 8
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	P. 7
Methods: Assignment of interventions (for controlled trials)			
Allocation:	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	P. 8
Sequence generation	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	P. 8
Allocation concealment mechanism	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	P. 7, P. 8

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Implementation	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	P. 8
Blinding (masking)	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	P. 8
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	P.9, P. 20 (Table 2)
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	n/a
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	P. 10
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	P. 9-10
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	P. 9-10
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	n/a
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	??
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	No plans for interim analysis
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	P. 8
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	P. 10
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	P. 10

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Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	n/a
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	P. 7, P. 10
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n/a
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	P. 7, P. 20 (Table 2)
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	P. 12
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	n/a
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	n/a
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	P. 10
	31b	Authorship eligibility guidelines and any intended use of professional writers	P. 12
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	n/a
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	n/a

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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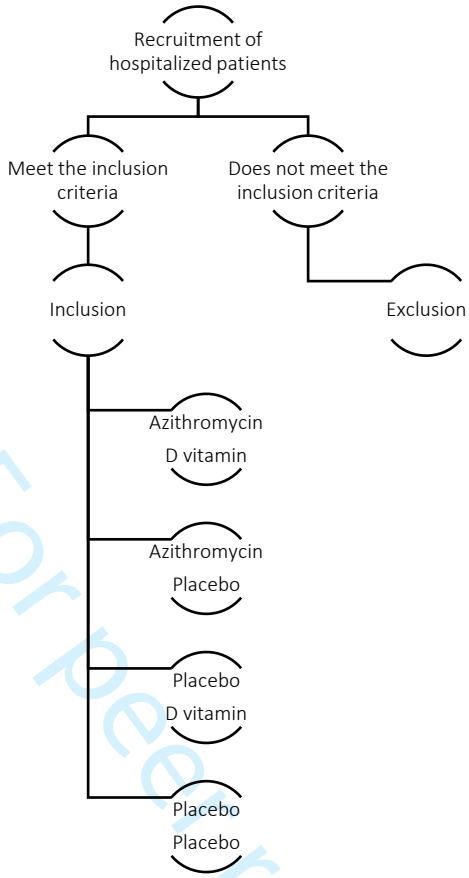

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Month = January (e.g.)	Date	1	2	3	4	5	6	…
Yes=x, No=0	Weekday	Mon	Tue	Wen	Thu	Fri	Sat	…
Healthy (i.e., no lung symptoms, no asthma medication). If yes, you do not have to fill in more.								
Lung symptoms	Cough							
	Shortness of breath							
	Wheeze							
Treatment of lung symptoms - Acute	Blue spray*							
	Oral steroid course**							
Treatment of lung symptoms - Preventive	Brown spray***							
	Asthma pill****							
Child's sick leave from daycare								
Possible adverse events								
If yes describe*****								

**SABA. **OCS. ***ICS. ****LTRA. ****Possible to write text.*

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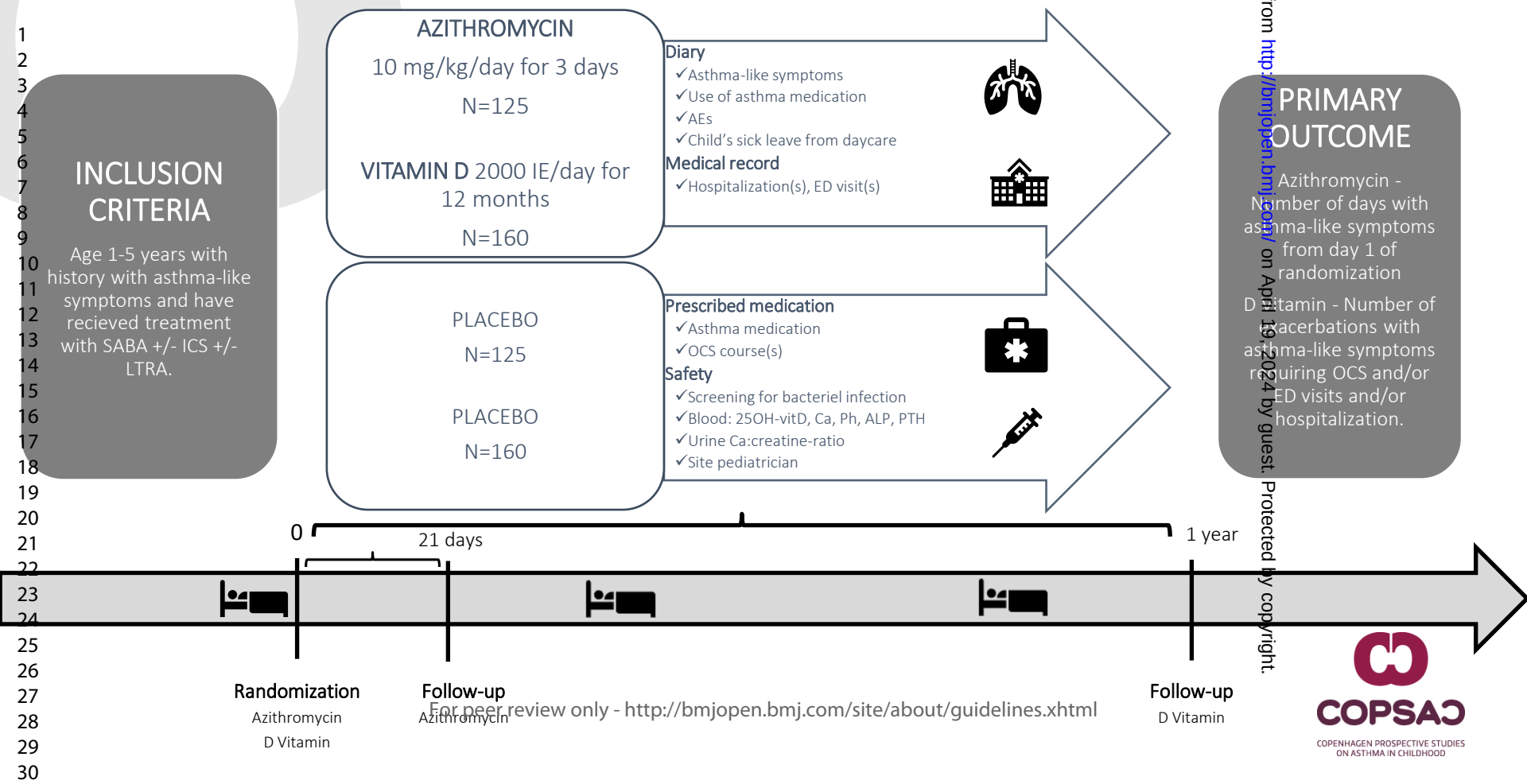

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Azithromycin and high-dose vitamin D for treatment and prevention of asthma-like episodes in hospitalized preschool children: study protocol for a combined double-blind randomized controlled trial

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Primary Subject Heading:	Paediatrics
Secondary Subject Heading:	Immunology (including allergy), Nutrition and metabolism, Pharmacology and therapeutics, Respiratory medicine

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Title

Azithromycin and high-dose vitamin D for treatment and prevention of asthma-like episodes in hospitalized preschool children: study protocol for a combined double-blind randomized controlled trial

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2 tables and 3 figures

Abbreviations:
AE = Adverse event
ALP = Alkaline Phosphatase
BMI = body mass index
Ca = Calcium

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COPSAC = Copenhagen Prospective Studies on Asthma in Childhood
ED = Emergency department
EFSA = European Food Safety Authority
GCP = Good Clinical Practice
ICS = Inhaled corticosteroids
IgE = immunoglobulin E
LTRA = Leukotriene receptor antagonist
OCS = Oral corticosteroids
PCA = Principal component analysis
PCR = Polymerase chain reaction
Ph = Phosphorus
PLS-DA=partial least squares-discriminant analysis
PTH = Parathormone
RCT = Randomized controlled trial
RTI = Respiratory tract infection
UL = Tolerable Upper Intake Level
SABA = short-acting beta-agonists
SD = Standard deviation
sPLS-DA = Sparse partial least squares discriminant analysis
25[OH]D = 25-hydroxyvitamin D

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Abstract

Introduction

Previous randomized controlled trials (RCTs) suggest antibiotics for treating episodes of asthma-like symptoms in preschool children. Further, high-dose vitamin D supplementation has been shown to reduce the rate of asthma exacerbations among adults with asthma, while RCTs in preschool children are lacking. The aims of this combined RCT are to evaluate treatment effect of azithromycin on episode duration and the preventive effect of high-dose vitamin D supplementation on subsequent episodes of asthma-like symptoms among hospitalized preschoolers.

Methods and analysis

Eligible participants are 1–5-year-old children with a history of recurrent asthma-like symptoms, hospitalized due to an acute episode will be randomly allocated 1:1 to azithromycin (10 mg/kg/day) or placebo for 3 days (n=250). Further, independent of the azithromycin intervention, participants will be randomly allocated 1:1 to high-dose vitamin D (2000 IU/day + standard dose 400 IU/day) or standard dose (400 IU/day) for 1 year (n=320). Participants are monitored with electronic diaries for asthma-like symptoms, asthma medication, adverse events, and sick-leave. The primary outcome for the azithromycin intervention is duration of asthma-like symptoms after treatment. Secondary outcomes include duration of hospitalization and anti-asthmatic treatment. The primary outcome for the vitamin D intervention is the number of exacerbations during the treatment period. Secondary outcomes include time to first exacerbation, symptom burden, asthma medication, and safety.

Ethics and dissemination

The RCTs are approved by the Danish local ethical committee and conducted in accordance with the guiding principles of the Declaration of Helsinki. Danish Medicines Agency has approved the azithromycin RCT and is monitored by the local Unit for Good Clinical Practice. The vitamin D RCT has been reviewed and is not considered a medical intervention. Results will be published in peer-reviewed journals and presented at international conferences.

Trial registration number

https://clinicaltrials.gov, NCT05028153, NCT05043116.
https://eudract.ema.eurupa.eu, 2020-004420-42.
Danish local ethical committee, H-20065249, H-20066827.

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Strengths and limitations of the study
• (+) This is a large combined RCT consisting of two independent interventions, which evaluate if a 3-days course of azithromycin (10 mg/kg/day) compared to placebo reduces the duration of asthma-like episodes in hospitalized preschool children, and if high-dose vitamin D supplementation (2000 IU/day+ standard dose 400 IU/day) for 1 year compared to standard dose (400 IU/day) reduces the subsequent number of asthma-like episodes requiring oral corticosteroids and/or emergency department visits and/or hospitalization.
• (+) If the interventions are proven effective, this RCT could have a huge impact on pediatric asthma management as the findings can be directly implemented in clinical practice and thereby address an unmet clinical need for treating and preventing recurrent asthma-like episodes in young children.
• (+) The children are carefully monitored with electronic diary recordings of asthma-like symptoms, anti-asthmatic treatment, and adverse events assuring robust and clinically relevant efficacy and safety endpoints.
• (+) Exploratory effect modifiers in both study arms include the airway immune profile, respiratory pathogens, airway microbiome assessments, blood eosinophil count, total-IgE and specific IgE levels and genetic markers, which enable analyzing underlying mechanisms of treatment effects and whether there are specific phenotypes or episode characteristics, where azithromycin and vitamin D treatment are more effective.
• (-) The azithromycin intervention could raise concern regarding antibiotic resistance and microbial derangements, which will be evaluated.

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INTRODUCTION

Childhood asthma is often preceded by recurrent episodes of asthma-like symptoms during the first years of life [1]. Severe episodes with asthma-like symptoms cause a high rate of emergency department (ED) visits and often require hospitalization [2,3] with high socioeconomic demands [4]. Typical triggers for asthma-like symptoms are respiratory tract infections (RTIs) of both viral and bacterial etiology [5,6], exercise, allergens, pollutants, and tobacco smoke. Exacerbations in preschoolers are most often triggered by RTIs [7] and have a poor response to oral corticosteroids (OCS) that are one of few options for treating exacerbations in this age group [8]. Preventive treatment of asthma in preschoolers is maintenance inhaled corticosteroids (ICS) as monotherapy and/or an oral leukotriene receptor antagonist (LTRA), which is effective for achieving daily symptom control, whereas many children still experience exacerbations requiring OCS and/or hospitalization [9]. Currently, there are no interventions aiming at reducing the high morbidity associated with acute exacerbations, which is a major unmet clinical need in this age group of vulnerable young children [10].

Azithromycin for treatment of asthma-like episodes

Antibiotics are not recommended for treating exacerbations of asthma-like symptoms in young children, although they are commonly used for this purpose [6,11]. As exacerbations of asthma-like symptoms can be triggered by bacterial RTIs, we previously conducted a randomized controlled trial (RCT) of azithromycin vs. placebo including 158 episodes of asthma-like symptoms in 72 children from the $COPSAC_{2010}$ cohort aged 12-36 months with a history of recurrent asthma-like symptoms [12]. The RCT showed that 3-days azithromycin (10 mg/kg/day) reduced the episode duration by 63% compared to placebo. Furthermore, the airway microbiota and airway immune responses during the episode was shown to predict children, who benefitted from the treatment [13,14].

A comparable American RCT included 443 children aged 12-71 months with a history of severe episodes with asthma-like symptoms showing that 5-days azithromycin (12 mg/kg/day) reduced the risk of progression from mild to severe lower respiratory tract illness with need for OCS by 36%, but episode duration was not reported [15]. A Canadian RCT included 222 children aged 12-60 months from an ED, admitted with acute asthma-like symptoms showing no effect on the duration of symptoms from 5-days azithromycin course (10 mg/kg/day) [16]. This study differed from the American RCT and our previous RCT as the children did not have a history of recurrent asthma-like symptoms. However, a sub-analysis among children with recurrent asthma-like symptoms showed similar results [16], but had a low number of subjects in the subgroup.

High-dose vitamin D for prevention of exacerbations

In children with asthma, vitamin D insufficiency is prevalent year-round and may increase the risk of exacerbations [17,18]. A recent systematic review and meta-analysis, which included several RCTs of vitamin D supplementation in children and adults showed that vitamin D significantly reduced exacerbations requiring OCS by 36% (n=658 adults; 22 preschoolers) and ED visits or hospitalizations by 61% (664 adults; 277 school-aged children; 22 preschoolers) compared to supplementation with 400 IU/day [19]. The systematic review underlines the shortage of RCTs in children, especially in preschoolers, where the largest preventive effect is to be expected as vitamin D has been shown to reduce the risk of RTIs [20], which are the main trigger of exacerbations in young children. Moreover, a RCT in adults (n=8) has suggested that high-dose Vitamin D (2000 IU/day) versus standard dose (400 IU/day) influences the expression several genes in white blood cells affecting a wide variety of biologic functions including genes involved in immune function [21].
Currently, there are 8 published pediatric vitamin D RCTs with respiratory outcomes [22], but only two includes preschoolers. The first is a safety pilot study of the DIVA RCT (NCT03365687) randomizing 22 preschool-aged children to an oral bolus of 100.000 IU plus 400 IU/day for 6 months

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or 400 IU/day. This was safe and raised serum 25-hydroxyvitamin D (25[OH]D) ≥75 nmol/L, but was underpowered to show differences in unplanned healthcare attendances due to acute wheeze [23]. The second is an American RCT investigating 12 months maintenance vitamin D repletion (oral 400 IU/day) vs. rapid repletion (oral 400 IU/day + intramuscular (IM) injection of either 300,000 IU (< 5 years of age) or 600,000 IU (> 5 years of age)) to children 2-14 years of age (mean age 6 years, 231 completers) with moderate-to-severe asthma exacerbations and 25[OH]D ≤25 ng/mL to prevent subsequent exacerbations [24]. Rapid compared to maintenance repletion significantly reduced unplanned visits for asthma exacerbations among children with baseline levels of 3 to 11 ng/mL during the initial 3 months of treatment, but not thereafter [24].
Apart from the safety pilot study of DIVA RCT and study by Alansari et. al. there are no other published RCTs of vitamin D supplementation to preschoolers with asthma-like symptoms. Though, the DIVA study on "Vitamin D in prevention of exacerbations of asthma in preschoolers" are currently recruiting participants, and is aiming to recruit 865 children [23]. Therefore, RCTs in preschoolers are needed to establish whether high-dose vitamin D supplementation can prevent exacerbations with asthma-like symptoms requiring OCS and/or ED visits and/or hospitalization [25].

It is important to investigate potential mechanisms of both azithromycin and high-dose vitamin D to be able to pinpoint specific phenotypes and episode characteristics, where treatment may be most effective.

Objectives

The main objective of the azithromycin arm of the study is to evaluate if 3-days azithromycin treatment (10 mg/kg/day) compared to placebo reduces the current episode duration.
The main objective of the vitamin D arm is to investigate whether high-dose supplementation (2000 IU/day + standard dose (400 IU/day) compared to standard dose (400 IU/day) reduces the number of subsequent episodes requiring OCS and/or ED visits and/or hospitalization. This will be studied among hospitalized children aged 1-5 years with a history of recurrent asthma-like symptoms.

METHODS

This study is a combined double-blind RCT with two independent interventions. Children, who meet the inclusion criteria for both interventions, will be asked to participate in one treatment arm consisting of azithromycin versus placebo and a second arm of high dose vitamin D (2000 IU/day + standard dose 400 IU/day) versus standard dose (400 IU/day) (see Figure 1). The study is coordinated by researchers at the Copenhagen Prospective Studies on Asthma in Childhood (COPSAC) research unit at Herlev-Gentofte Hospital. Planned start to recruit participants is February 2022, and we estimate a 3 years-timespan for recruiting all participants (i.e., February 2024).

Participants, intervention, and outcomes

Study setting

The study will be conducted at three pediatric departments in Zealand, Denmark: Herlev-Gentofte University Hospital, Hvidovre University Hospital, and Slagelse Hospital. Additional pediatric departments will be included if needed.

Eligibility criteria: inclusion/exclusion

The inclusion criteria for participation in both study arms are: (i) hospitalization due to an episode of asthma-like symptoms, (ii) age 12-71 months, (iii) a medical history of episode(s) with asthma-like symptoms and treatment with short-acting beta-agonists (SABA) as monotherapy, or in combination with ICS and/or LTRA. Further, parents must have fluent Danish skills. The specific inclusion and exclusion criteria for participation in the two study arms are listed in Table 1.

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The exclusion criteria aim to minimize the risk of allergic reactions and of including children, who suffer from pneumonia or other bacterial infections, rickets, moderate to severe vitamin D deficiency (25[OH]D < 25 nmol/l), as the Danish Pediatric Society's guidelines regarding treatment of vitamin D deficiency recommend 800 IU/day when 25[OH]D <25 nmol/l [26], and risk of vitamin D intoxication.

Interventions

In the azithromycin arm the children are randomized to a 3-days course of azithromycin (10 mg/kg/day) or placebo. The interventions consist of powder for oral suspension 40 mg/mL azithromycin or placebo in identical looking 22,5 mL packings. The parents will report their child's asthma-like symptoms defined as wheeze or whistling sounds, breathlessness, or troublesome cough severely affecting the well-being of the child, use of asthma medication, adverse events (AEs), and sick leave via a link sent per text messaging once daily for 21 days via the secure system Research Electronic Data Capture (REDCap).

In the vitamin D arm, the children are randomized to one daily oral dose of vitamin D supplementation of 2000 IU (~50 µg) or an identical lookalike placebo for 1 year. We recommend all parents to continue supplementing their child with 400 IU/day (~10 µg) as this is recommend by Danish Health Authority [27]. Therefore, the intervention group will receive a total intake of 2400 IU/day (~60 µg/day). The intervention preparation consist of D-Pearls (i.e., small and soft ox gelatin capsules 1000 IU/D-Pearl) or placebo in identical looking D-Pearls produced by Pharma Nord. To avoid seasonal variations in 25[OH]D levels at baseline and the seasonal variation in RTIs, the intervention is given for 12 months. The parents will report their child's asthma-like symptoms, use of asthma medication, AEs, and sick leave via a weekly REDCap link, where they can fill out daily information from the prior week (See Figure 2).

Outcomes

The primary outcome in the azithromycin study arm is the duration after randomization of the episode with doctor-diagnosed asthma-like symptoms, so severe that the symptoms have led to hospitalization.

Asthma-like symptoms are defined as wheeze or whistling sounds, breathlessness, or troublesome cough severely affecting the well-being of the child, which is a validated method to monitor asthma-like symptoms [28–30], and used several times in our clinical observational studies of young children [28,29,31–33], and in two RCTs including our previous azithromycin RCT [12,34]. Secondary outcomes are: (i) symptom burden; (ii) length of hospitalization; (iii) intensity of episode treatment; (iv) leave from daycare; (v) health economic benefits; (vi) gut microbiome and resistance profiles (assessed by metagenomic sequencing for composition, diversity, and abundance of bacterial taxa and antibiotic resistance genes [35] at baseline, 3 weeks, 3 months and 1 year after treatment with azithromycin. Evaluation of effect modification by (vii) airway microbiome (virus detection by polymerase chain reaction (PCR) and bacterial 16S rRNA sequencing), and (viii) airway immune profile. Further, safety by (ix) AEs for three weeks.

The primary outcome in the vitamin D study arm is the number of episodes with asthma-like symptoms requiring OCS and/or ED visit and/or hospitalization over a 12-month period after randomization. Secondary outcomes are: (i) time to first exacerbation; (ii) number of episodes with asthma-like symptoms; (iii) length of hospitalization; (iv) symptom burden between exacerbations; (v) treatment during exacerbations; (vi) step-down asthma maintenance therapy; (vii) health economic benefits. Evaluation of effect modification by (viii) baseline 25[OH]D level; (ix) genetic

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variation and expression in the vitamin D pathway, *e.g.,* VDR and VDBP; (x) genetic variation and expression in known childhood asthma loci, *e.g.,* 17q21; (xi) airway microbiome; (xii) airway immune profile and (xiii) atopic status, *i.e.,* blood eosinophil count, total immunoglobulin E (IgE) and specific-IgE levels towards aeroallergens (xiv) number of asthma-like episodes the previous year; (xv) requiring vs. not requiring OCS the last year. Further, safety profile by clinically significant; (xvi) hypercalciuria; (xvii) hypercalcaemia; (xviii) 25[OH]D>250 nmol/L; and (xix) AEs for one year. Furthermore, (xx) prevention of and possible reduction of symptom burden from COVID-19.

Participant timeline

Recruitment of participants will start February 2022 and is expected to last 3 years. The study will be completed when at least 250 participants are recruited for the azithromycin study arm and 320 participants for the D vitamin study arm. Study duration per participant for the azithromycin arm is 21 days, the first 3 days with treatment, and fecal samples are further collected at inclusion, 21 days, 3 months and 1 year after intervention. Study duration per participant for the vitamin D arm is 1 year (See Figure 3). The estimated completion of the study is February 2025.

Recruitment

A doctor and a nurse from the research team will contact the pediatric wards every morning on weekdays to screen for newly admitted patients eligible to include in the study. If this is the case, layperson summaries, including participant information and the informed consent form, which describes the experiment and its procedures, will be handed to the parents. If the parents after reading the layperson summaries are interested in participating, the research team will make an appointment with the family on the same day or latest within 3 days. Inclusion is only possible while the child is hospitalized. We will inform orally about the studies purposes, content, risks, form etc. Here, the parents will have the possibility to ask questions before finally deciding to participate.

Adverse events

Azithromycin is a commonly used macrolide in children and is shown safe in several RCTs including the RCT performed in the $COPSAC_{2010}$ cohort [12,36,37]. Of known AEs the most frequent are gastrointestinal (*i.e.,* diarrhea, nausea, vomiting), whereas other AEs are rare (*e.g.,* allergic reaction). AEs due to vitamin D treatment are rare. The European Food Safety Authority (EFSA) has provided Tolerable Upper Intake Levels (ULs) of vitamin D for children of all ages, concluding that for 1 to 10-year-olds, UL is 2000 IU/day [22]. Recently, the TARGet Kids! Collaboration conducted a RCT randomizing 703 healthy 1-5-year-old children to 2000 IU/day or 400 IU/day for a minimum of 4 months to reduce RTIs, where no vitamin D related AEs were reported [38]. Based on above mentioned studies the intervention dose chosen in this RCT is 2000 IU/day + standard dose 400 IU/day. Potential Aes of high-dose vitamin D treatment include (i) hypocalcemia; and (ii) hypercalcaemia/calciuria. These Aes are rare and are assessed at inclusion, after 3 months, and at a 1-year follow-up visit by measuring 25[OH]D, Calcium (Ca), Phosphorus (Ph), Alkaline Phosphatase (ALP), Parathormone (PTH) and urine Calcium:Creatinine ratio, which will be evaluated by an independent pediatrician.

If a child unexpectedly develops symptoms of severe Aes, such as intoxication or allergic reaction, treatment will be stopped immediately, randomization unblinded, and the participant will be referred for assessment and treatment. Further, if a participant is diagnosed with moderate to severe vitamin D deficiency (25[OH]D <25 nmol/l) the child will be excluded from the study and referred to their general practitioner for a higher supplementation dose according to Danish Pediatric Society's recommendations [26].

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Sample size

The main outcome in the azithromycin study arm is the duration of the episode with asthma-like symptoms in days; *i.e.* non-normally distributed. Due to some observations of zero days duration, we will add a pseudocount of 1-day duration for transformation of all durations: log(duration+1) = β_0 + R*β_1 + error, where R is the intervention. Based on the $COPSAC_{2010}$ RCT duration of symptoms [12], we expect a standard deviation (SD) of log(duration +1) = 1.04. With a power of 80% and a two-tailed alpha (α) of 5%, a delta (λ) in geometric mean ratio of 1.5, corresponding to a 50% reduction in symptoms duration, we need a sample size of n = 230 children randomized in two groups on a 1:1 and expecting a drop out of 10%. To increase power, we aim to recruit 250 children (125/arm).
The main outcome in the vitamin D study arm is the number of episodes with asthma-like symptoms requiring OCS and/or ED visit and/or hospitalization over a 12 months period (λ), which is estimated at 0.94/child in the placebo arm based on previous RCTs with ICS [39]. A sample size of 145 children per arm will provide 80% power with a two-tailed α of 5% to detect a 35% relative reduction in the mean number of events (*i.e.*, λ=0.94 in the placebo vs. λ=0.611 in the intervention group), based on a previous observed rate ratio of 0.64 for exacerbations requiring OCS in a meta-analysis [19]. We expect a 10% drop out and therefore aim to recruit 320 children (160/arm).

Blinding and randomization

The participants will be randomly allocated 1:1 in both study arms, independently. The randomization in both study arms will be done at Glostrup Pharmacy by a computer-generated list of random numbers in blocks of varying size (i.e., 4, 6 or 8). Copies of the randomization code will be kept in sealed envelopes at the research site and at the Glostrup Pharmacy.
Treatment compliance in the azithromycin study arm is ascertained by parents checking a REDCap link checkbox after administration during the first three days.
Treatment compliance in the vitamin D study arm is ascertained by parents weekly via a REDCap link, where there is a checkbox for how many days the current week D-pearls were given and by counting the returned blister packs (both used and unused) at the 1-year follow-up visit.
All participants, parents, and members of the research team are blinded to group allocation. Efficacy and safety analyses will be performed under allocation concealment. Both study arms will remain double-blinded until all children have been recruited and throughout the data validation and primary outcome analysis phases.

Data collection, management, and analyses

Data collection methods

Through an interview with the child and parent(s) data on baseline health including, data on former episode(s) with asthma-like symptoms, current and former asthma medication, atopic status, and data on environment and sociodemographic will be collected and directly entered in the REDCap trial database (See Table 2 for details recorded). Moreover, we will extract data on prescribed medication and information from the child's medical records from birth and 4 years ahead from date of inclusion. We will monitor asthma-like symptoms, defined as wheeze or whistling sounds, breathlessness, or troublesome cough severely affecting the well-being of the child, via diary data registered online via the REDCap system. The parents will report daily for 21 days their child's asthma-like symptoms, use of medication, possible AEs, and sick leave. A link for this registration is sent once daily via REDCap. If the child also participates in the vitamin D study arm, the parents continue to fill out the diary once weekly for 1 year. The participants included in the azithromycin study will deliver a blood sample at baseline, measuring C-reactive protein and white blood cell count. Fecal samples will be collected at baseline, 3 weeks, 3 months, and 1 year after inclusion. Both studies include biosamples for measuring airway microbiota, pathogenic bacteria, vira, and the

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airway immune profile. The vitamin D study arm also includes samples from the nose for gene expression of the upper airway. Further, in the vitamin D study arm at inclusion and after 3 months (i.e., at their local hospital) a blood sample and a urine sample will be taken for measuring 25[OH]D, Ca, Ph, ALP, PTH and urine Calcium:Creatinine ratio, which will be evaluated by an independent pediatrician for signs of vitamin D intoxication, and vitamin D insufficiency.

Further, they will attend a clinical follow-up visit at the COPSAC research clinic 1 year after enrolment for collection of a variety of bio samples, screening for AEs, and interviewing the parent(s) regarding the recent years' exacerbations, medication use, and step-up/step-down in preventive asthma medication.
The planned study procedures are listed in Table 2. Further, a more in-depth description of the methods for sampling and assessments of the biosamples is given in the Supplemental material.

Statistical methods

An intention-to-treat analysis will be carried out with all randomized children by treatment group.

Azithromycin study

We will analyze the duration of an episode of asthma-like symptoms and SABA use after treatment with Quasi-Poisson regression models. This type of regression is ideal for modelling counts, such as duration because it captures both skewness and variance heterogeneity and provides an easy-to-interpret quantification of effects as relative change in mean counts. Further, we will analyze time to resolution of asthma-like symptoms using survival analysis, e.g., Cox regression or accelerated failure time method, though specific model chosen will be dependent on model fit. We will evaluate potential effects of factors such as recruiting center and age of the child by inclusion of these in the models.
We will analyze baseline factors potentially modifying the treatment effect such as airway immune profile, airway microbial colonization, and clinical appearance including current use of anti-asthmatic treatment as interaction terms in the models. The modifying effect of airway immune profile and airway microbial colonization are investigated to confirm earlier findings.

This is planned as we previously showed that lower levels of TNF-alpha and CCL22 and higher IL-10 in the upper airway predicted better treatment response to azithromycin [14]. Furthermore, we showed that the composition of the airway microbiota during an acute episode modified the effect of azithromycin treatment with high richness or high relative abundance of *Veillonella* and *Neisseria* being associated with a better treatment effect of azithromycin [13].

We will apply unsupervised compositional methods such as principal component analysis (PCA) and alpha- and beta-diversity measures for the analysis of inflammatory markers and microbiota as well as univariate single variable testing and additionally supervised methods such as partial least squares-discriminant analysis (PLS-DA) / sparse partial least squares discriminant analysis (sPLS-DA) to evaluate jointly contributing variables.

Safety analyses will include all children who receive the intervention, including those, if any, without a primary outcome measure.

Vitamin D study

We will analyze the number of exacerbations requiring OCS and/or ED visit and/or hospitalization per child during the 12-month follow-up using a Quasi-Poisson regression model to compute the incidence risk ratio.

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Six subgroup analyses are planned: 25[OH]D (<75 vs ≥75 nmol/L and 1st vs. 4th quartile), sex (male/female), atopy (sIgE ≤0.35 or >0.35 kUa/L), elevated blood eosinophil count (≤0.3 or >0.3 x10^9 cells/mL), body mass index (BMI) (Z-score ≤2 vs >2 SD), and ethnicity (Caucasian vs non-Caucasian). Specific ethnicities will be registered, and if possible, we will stratify analysis on ethnicity.
Effect modification analyses are planned for baseline airway immune mediator levels, respiratory pathogens (virus and bacteria), airway microbiome, and for genetic variations in the vitamin D pathway/metabolism and childhood asthma risk loci.
The time to first exacerbation requiring OCS and/or ED visit and/or hospitalization will be compared between groups using a cox regression model. Symptom/hospitalization duration during an exacerbation will be compared between groups using a Quasi-Poisson or general linear regression model. Similar analyses will be carried out for intensity of SABA and OCS use. Daily symptom burden between exacerbations will also be compared using a Poisson or general linear regression model.
The Chi-square test or Mantel-Haenszel method will serve to compare categorical outcomes, including the number of children with ≥1 episode of clinically significant hypercalciuria, hypercalcemia, elevated serum 25[OH]D, and AEs.

No adjustment for multiple outcomes is planned in any of the study arms.

Data management and monitoring

Acquired data is stored in the secure REDCap system provided by the Capital Region of Copenhagen. The data are directly entered electronically, both by researchers and the parents. The azithromycin study arms will be monitored by the unit of Good Clinical Practice (GCP), Bispebjerg Hospital. GCP will oversee randomization, data management, progress monitoring and all analyses. A combination of remote monitoring activities and routine monitoring visits are conducted to ensure that each site adheres to the study protocol, GCP guidelines and data collection completeness.

Ethics and dissemination

The studies are conducted in accordance with the guiding principles of the Declaration of Helsinki and are approved by the Danish ethical committee (H-20065249, H-20066827). The Danish Medicines Agency has approved the azithromycin study arm (EudraCT number: 2020-004420-42). The vitamin D intervention has been reviewed as well and is not considered to be a medical intervention. All families will receive written information about the study prior to participation and both parents will give verbal and written informed consent before enrolment. Acquired data is stored in the secure REDCap system. No provision is given to the participants, and they have the possibility to withdraw from the study at any time.
No participant identifiers will be used in the dissemination of this research. Results will be disseminated to the medical community via national/international conferences and publications in peer-reviewed journals, to lay people via social and public media, and to families of preschoolers with asthma by involving patient organizations and networks. All results, negative as well as positive, will be published. Further, the results will be available on our websites: www.copsac.com and www.dbac.dk. The studies are registered at https://clinicaltrials.gov (NCT05028153, NCT05043116) and the azithromycin study arm at https://eudract.ema.eurupa.eu (2020-004420-42).

Public and Patient Involvement

No patients were involved in setting the research question or the outcome measure, nor are they involved in developing plans for recruitment, design, or implementation of the study. No patients

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will be asked to advise on interpretation or writing up of results. We will disseminate the results of the research to study participants and the public.

DISCUSSION

This paper describes the design and methodology of a combined RCT, consisting of two independent interventions with the overall aim to improve both secondary prevention (high-dose vitamin D) and tertiary prevention (azithromycin) of asthma-like symptoms in preschoolers. This has the potential to fill in a huge medical need as the prevalence of childhood asthma has increased markedly in the last decades [2] and there are limited treatment possibilities for both secondary and tertiary prevention of exacerbations.

We hypothesize that azithromycin treatment shortens episode duration of asthma-like symptoms so severe that the symptoms have led to hospitalization of preschool children. If successful, the project will provide evidence of azithromycin as a potential standard treatment for recurrent acute asthma-like episodes requiring hospitalization. A secondary aim is to assess the individual responses to azithromycin treatment based on the airway immune profile and the airway microbiome. Previously, we investigated levels of 18 cytokines and chemokines *in vivo* in the upper airway to assess the value of these mediators for predicting treatment response to azithromycin. Levels of TNF-α, IL-10 and CCL22 were found to predict treatment response [14]. Further, in the same study, we also found that the composition of the airway microbiota during the acute episode modified the treatment effect of azithromycin. A high bacterial richness was associated with a better treatment effect and high relative abundance of several individual bacterial taxa similarly increased the treatment effects [13]. If replicated, these results may pave a path for a personalized treatment strategy from immunological and/or microbial profiles, so only children, who will benefit from azithromycin will be treated. Thereby, we aim to pinpoint the children, which will have the best treatment effect to lower the number of children treated with azithromycin.

The azithromycin study can raise concerns regarding antibiotic resistance. However, the intervention only targets children with recurrent asthma-like episodes, who currently receive or have received anti-asthmatic medication, which narrows the group, where the treatment will be relevant. A focus on personalized treatment in the future will further narrow down the group, where azithromycin is indicated. Finally, we have previously reported that azithromycin treatment only leads to short-term deranged gut microbiota (*i.e.,* 21 days after intervention), which was normalized later in childhood (*i.e.,* 1-3 years after intervention) [40].

We hypothesize that high-dose vitamin D treatment will lead to more effective secondary prevention of episodes with asthma-like symptoms in preschoolers currently receiving or having received anti-asthmatic medication. We will investigate potential mechanisms of action to elucidate the protective role of vitamin D, which includes effect modification analyses of airway immune mediator levels, the airway microbiome, and genetic variations and gene expression in the vitamin D pathway/metabolism and known childhood asthma risk loci. This strategy has the potential to highlight specific phenotypes of asthma and episode characteristics, where vitamin D supplementation is most effective. Importantly, vitamin D supplementation has been found to reduce the risk of RTI in a subgroup analysis within a meta-analysis of children aged 1–16 years by 40% (n = 513 children) [20]. This suggests that vitamin D may be particularly beneficial for prevention of episodes in preschoolers, where RTI is the main trigger of exacerbations. This effect on RTIs further suggests that vitamin D has its impact via the immune system. Hence, exploring the role of baseline vitamin D levels and the impact of vitamin D on airway immune mediators would enhance our understanding of mechanisms of action.

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This combined placebo-controlled, double-blind, RCT with two independent interventions has several strengths, which include (i) enrolment of hospitalized preschoolers with asthma-like symptoms irrespective of atopy, phenotype and baseline 25[OH]D (vitamin D arm); (ii) a short-term (*i.e.* 3 days for azithromycin), safe, and easy single daily dosing without bolus treatment for vitamin D arm; (iii) a 12-months intervention for vitamin D ensuring that the study period continues throughout winter and covers the peak incidence of exacerbations and RTIs and the seasonal decline in 25[OH]D; (iv) primary outcomes that address unmet clinical and societal needs; (v) exploration of potential mechanisms of action to direct personalized treatment; (vi) safety profile documentation; and (vii) interventions that can easily be implemented in clinical practice. RCTs are necessary as a basis for evidence-based guidelines before being able to (i) implement azithromycin as part of the tertiary prevention of asthma in preschool children and (ii) implement vitamin D supplementation as a part of secondary prevention of asthma in preschool children.

The project will be performed by the research team at COPSAC in collaboration with pediatric dept. at Herlev-Gentofte University Hospital, Hvidovre University Hospital, and Slagelse Hospital, Denmark.

Acknowledgements
We used the SPIRIT checklist when writing this report.

Contributors
BLKC, JS, HB, KB, LMH and JNK designed the studies. JNK was responsible for writing the first draft of the manuscript. UR were responsible for the data system REDCap and contact to the pharmacy and the GCP unit. JNK, BLKC, JS, UR, NVF, TM, LMH, AMS, KB, HB read and critically revised the manuscript.

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Competing interests
None declared.

Patient consent for publication
Not required.

Provance and peer review
Not commissioned, externally peer reviewed.

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Data sharing statement and ethical approval
The studies will record two datasets on in total 250 participants in the azithromycin randomized controlled trial (RCT) and 320 participants the vitamin D RCT, most likely participants will be included in both studies. We collect from interviews and medical records demographic-, growth-, birth data, data on atopic status, medication, and former episode(s) with asthma-like symptoms. Further, we collect bio samples, where data from laboratory analysis will be stored. Researchers are all blinded while the trials include and follow the participants. The data are stored in the secure system Research Electronic Data Capture. The studies are approved by the local ethical committee and reviewed by Danish Medicines Authority. Further, the studies are monitored by the local Unit for Good Clinical Practice. Data will be stored for 10 years from collection date and hereafter it will be destroyed. The data from clinical trials to be made available upon reasonable request.

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Tables

Table 1 *Overview of the Inclusion and exclusion criteria for azithromycin and vitamin D. BMI, body mass index; ICS, inhaled corticosteroids; LTRA, leukotriene receptor antagonist; SABA, short-acting beta-agonists; 25[OH]D, 25-hydroxyvitamin D.*

Inclusion Criteria	
Azithromycin and vitamin D study	• Hospitalization due to an episode of asthma-like symptoms • Age 12-71 months • Medical history of episode(s) with asthma-like symptoms and treatment with SABA as monotherapy, or in combination with ICS and/or LTRA
Exclusion Criteria	
Azithromycin and vitamin D study	• Clinical signs of pneumonia (tachypnoea: Respiratory rate >50 and/or fever: temperature >39°C and/or C-reactive protein >50) • Chronic lung disease (other than asthma/wheeze) • Impaired liver or kidney function • Neurological or psychiatric disorders • Congenital or acquired prolonged QT interval • Clinically relevant bradycardia, cardiac arrhythmia, or severe heart failure.
Azithromycin study	• Macrolide allergy
Vitamin D study	• Daily intake of vitamin D supplementation or receives a combination of vitamin and dietary supplements containing vitamin D > 400 IU/day (~10 µg/day) • Moderate to severe vitamin D insufficiency (i.e., 25[OH]D <25 nmol/l) • Malnourished (i.e., children >2 years - age-specific BMI less than the 3rd percentile and children <2 years - weight or height in relation to age are less than the 3rd percentile) • Newly arrived refugee/immigrant from regions with high incidence of rickets • Receives medication that alters calcium or Vitamin D absorption/metabolism

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Table 2 *Overview of data we will access at the time of inclusion and over study procedures. If the child is participating in both study arms the overlapping procedures will only be taken once. AEs, adverse events; ALP, Alkaline Phosphatase; BMI, body mass index; Ca, Calcium; ICS, inhaled corticosteroids; IgE, immunoglobulin E; LTRA, leukotriene receptor antagonist; OCS, oral corticosteroids; Ph, Phosphorus; PTH, Parathormone; REDCap, Research Electronic Data Capture; SABA, short-acting beta-agonists; 25[OH]D, 25-hydroxyvitamin D.*

Participant	Baseline	21 days	3 months	1 year	Description		References
Azithromycin study arm							
Fecal sample	X	X	X	X	Measurement of gut microbiota using 16S rRNA sequencing and antibiotic resistance genes assessed by metagenomic sequencing		[35,40]
Diary	X	X			Diary on asthma-like symptoms, medication, sick leave, and AEs is registered daily by parents via text messaging through REDCap		[12,28]
Hypopharyn-geal aspirate	X				Measurement of airway microbiota and viruses		[41]
Filter-paper sampling	X				Evaluation of immune mediator profiles in the upper airway epithelial lining fluid		[42]
Blood sample	X				Inflammatory markers (i.e., C-reactive protein, white blood cell count)		
Anthropo-metrics	X				Height, weight		
Physical examination	X				Assessment of fever, tachypnoea, chest recessions, wheezing, and lung and heart auscultation, and examination of the skin, ears, nose, and throat		
Interview	X				Baseline characteristic	Age, sex, ethnicity, address	
					Growth data	Weight, height, BMI z-score	
					Birth data	Gestational age, birth weight and length, delivery mode, siblings at birth.	
					Atopic status	Food allergies, allergic rhinoconjunctivitis, atopic dermatitis	
					Asthma medication	SABA, ICS, OCS, LTRA (current and former)	
					Former episode(s) with asthma-like symptoms	Time, numbers, treatment (incl. medication and emergency room visit(s), hospitalization(s), visit to general practitioner or treated at home)	
					Other relevant data	Daycare status (if yes - also start date), smoking in the home, animal in the home	
					Parents and sibling(s) atopic status	Asthma diagnosis, food allergies, allergic rhinoconjuctivitis, atopic dermatitis	
					Socioeconomic status	Parent's education level, parents age, parents' income.	
Vitamin D study arm							
Diary	X			X	Diary on asthma-like symptoms, medication, sick leave, and AEs is registered daily the first 21 days, afterwards weekly, by the parents, through text messaging via REDCap		[12,28]
Hypopharyn-geal aspirate	X			X	*		[41]
Filter-paper sampling	X			X	*		[42]
Nasal epithelial scrape	X			X	Gene expression		[43]
Urine sample	X		X	X	Calcium:Creatinine-ratio		
Anthropo-metrics	X				*		
Physical examination	X				*		
Blood sample	X		X	X	At inclusion and at the 1-year follow-up visit: 25[OH]D, Ca, Ph, ALP, PTH, C-reactive protein, white blood cell count. Gene expression and methylation in vitamin D pathways and childhood asthma risk loci. At 3 months after inclusion 25[OH]D, Ca, Ph, ALP, PTH. Total-IgE and specific-IgE towards inhalant allergens will be examined after 1 year.		
Interview regarding exhibitions	X				*		

**As in the Azithromycin study arm*

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Figure legend/caption

Figure 1 *Flow diagram of enrolment of patients. Patients who meet the inclusion criteria in both study arms (i.e., vitamin D and azithromycin) can participate in both studies independently of each other.*

Figure 2 *Diary the parents fill out on information on asthma-like symptoms, asthma medication use, AEs, and sick leave. Further, a checkbox for administration of azithromycin is added to diary during the first three days, and a checkbox for how many days the current week D-pearls were given. ICS, inhaled corticosteroids; LTRA, leukotriene receptor antagonist; OCS, oral corticosteroids; SABA, short-acting beta-agonists.*

Figure 3 *Overview of the study timeline for both study arms (i.e., 21 days in the azithromycin arm and 1 year in the vitamin D arm). AEs, adverse events; ALP, Alkaline Phosphatase; Ca, Calcium; ICS, inhaled corticosteroids; LTRA, leukotriene receptor antagonist; OCS, oral corticosteroids; Ph, Phosphorus; PTH, Parathormone; SABA, short-acting beta-agonists; 25[OH]D, 25-hydroxyvitamin D.*

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	ItemNo	Description	Page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	P. 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	P. 3, P. 11
	2b	All items from the World Health Organization Trial Registration Data Set	n/a
Protocol version	3	Date and version identifier	Headnote at all pages.
Funding	4	Sources and types of financial, material, and other support	P. 13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	P. 1, P. 13
	5b	Name and contact information for the trial sponsor	P. 1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	P. 13
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	P. 13
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	P. 5-6, P. 4 (box regarding strength and limitation)
	6b	Explanation for choice of comparators	P. 5-6
Objectives	7	Specific objectives or hypotheses	P. 6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	P. 6
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	P. 6

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Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	P. 6-7, P. 14 (Table 1)
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	P. 3,4,6
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	P. 8
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	P. 8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	P. 6, P. 14 (Table 1)
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	P. 7-8
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	P. 8
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	P. 9
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	P. 8
Methods: Assignment of interventions (for controlled trials)			
Allocation:	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	P. 9
Sequence generation	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	P. 9
Allocation concealment mechanism	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	P. 8, P. 9
Implementation	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	P. 9
Blinding (masking)	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	P. 8

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Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	P.9-10, P. 15 (Table 2)
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	n/a
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	P. 11
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	P. 10-11
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	P. 10-11
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	n/a
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	??
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	No plans for interim analysis
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	P. 8
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	n/a
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	P. 11
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	n/a
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	P. 8, P. 11

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	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n/a
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	P. 7, 9, 11 P. 15 (Table 2)
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	P. 13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	n/a
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	n/a
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	P. 11
	31b	Authorship eligibility guidelines and any intended use of professional writers	n/a
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	n/a
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	n/a
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	n/a

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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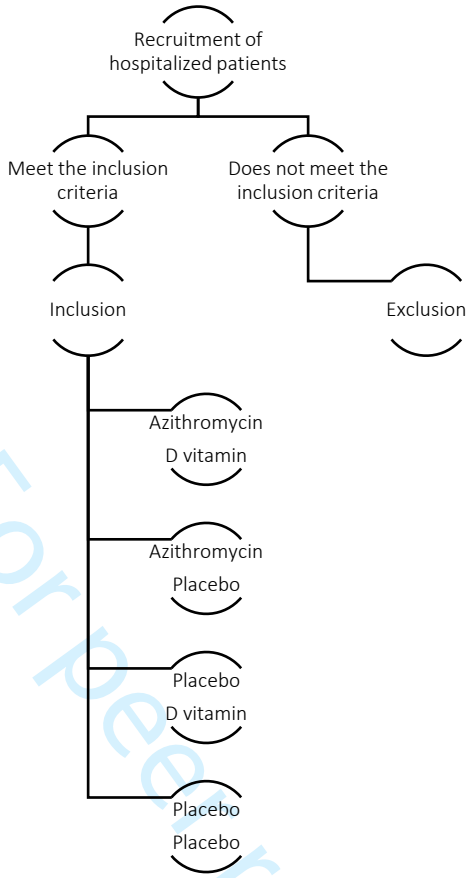

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Month = January (e.g.)	Date	1	2	3	4	5	6	...
Yes=x, No=0	Weekday	Mon	Tue	Wen	Thu	Fri	Sat	...
Healthy (i.e., no lung symptoms, no asthma medication). If yes, you do not have to fill in more.								
Lung symptoms	Cough							
	Shortness of breath							
	Wheeze							
Treatment of lung symptoms - Acute	Blue spray*							
	Oral steroid course**							
Treatment of lung symptoms - Preventive	Brown spray***							
	Asthma pill****							
Child's sick leave from daycare								
Possible adverse events								
If yes describe*****								

**SABA. **OCS. ***ICS. ****LTRA. ****Possible to write text.*

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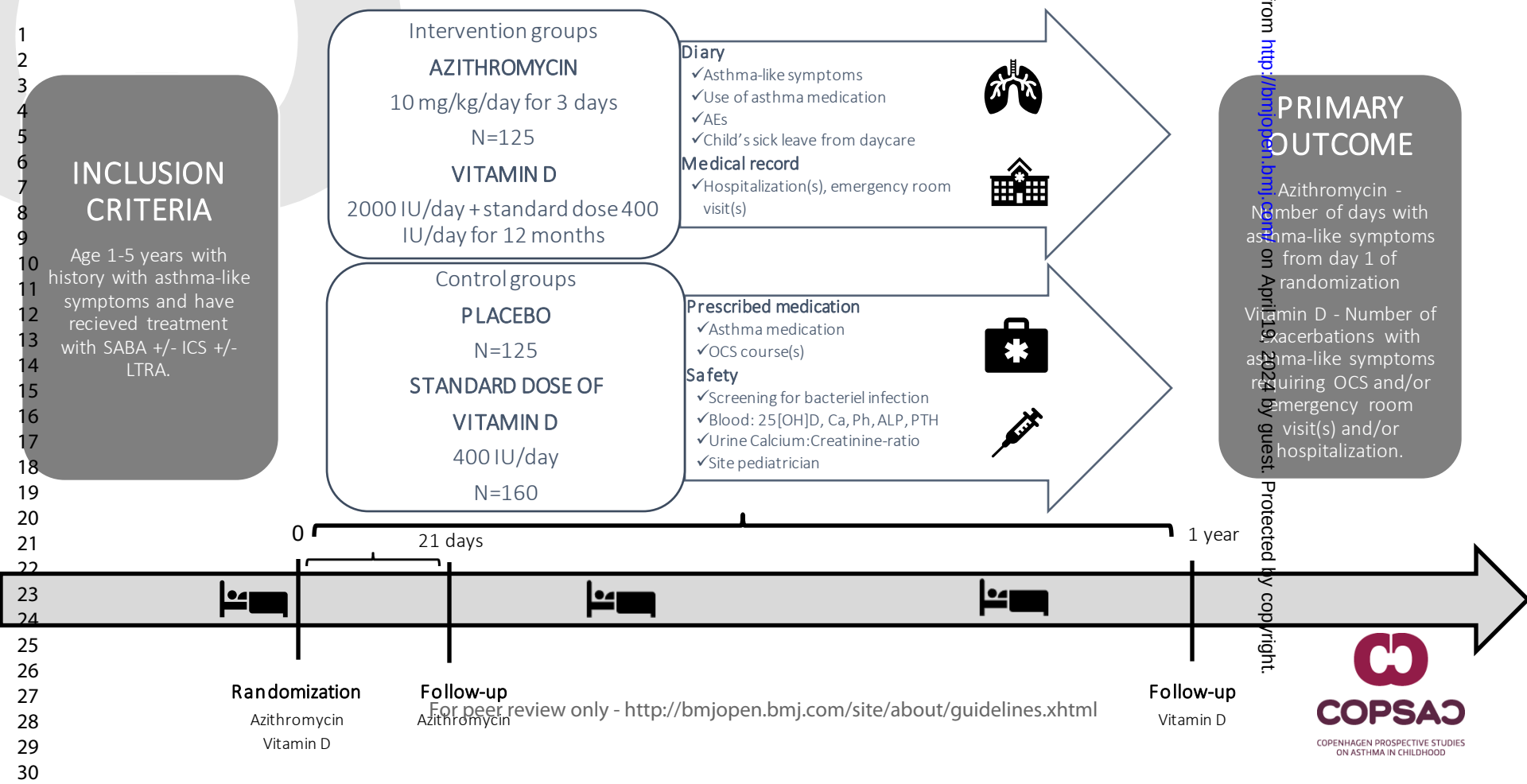
DOUBLE-BLIND, RANDOMIZED CONTROLLED TRIALS



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COPENHAGEN PROSPECTIVE STUDIES ON ASTHMA IN CHILDHOOD

Supplemental material:

Hypopharyngeal aspirates are planned to use a soft suction catheter passed through the nose into the hypopharynx. We plan to analyze the samples by culture independent 16S rRNA sequencing as previously detailed [1], but with species annotation by sequencing of the hypervariable V3-V4 region instead of the V4 region in our previous study. Further, the aspirates will be analysed for viral identification with PCR (i.e., rhinoviruses, respiratory syncytial virus (RSV), and enteroviruses) [2].

Fecal samples will be analyzed by shotgun metagenomic sequencing, which apart from species level taxonomic resolution additionally will provide information on all resistance genes in the sample [3].

Upper airway mucosal lining fluid will be collected with a pair of 3- 3 15-mm strips of filter paper (Accuwik Ultra; fibrous hydroxylated polyester sheets, cat no. SPR0730, Pall Life Sciences, Portsmouth, Hampshire, UK; this product is no longer manufactured, but Leukosorb from Pall Life Sciences is an alternative) inserted onto the anterior part of the inferior nasal turbinate of both nostrils (see online video of the sampling procedure; www.copsac.com), and left for 2 minutes. Afterwards, the filter papers will be frozen at 2808C and stored until analysis. The levels of IFN-g, IL-1b, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13, tumor necrosis factor (TNF)-a, CXCL8 (IL-8), CCL11 (eotaxin-1), CCL26 (eotaxin-3), CXCL10 (IP-10), CCL2 (MCP-1), CCL13 (MCP-4), CCL22 (MDC), CCL4 (MIP-1b), and TARC (CCL17) in the extracted upper airway mucosal lining fluid will be analyzed in duplicate using the Ultrasensitive Meso Scale Discovery Multi-spot Human TH1/TH2 10-Plex cytokine assay and 9-plex chemokine assay (Meso Scale Discovery, Gaithersburg, MD), using the Sector Imager 6000 (Meso Scale Discovery) [4].

Nasal epithelial scrape samples will be taken from both nostrils, and RNA will extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and purified further by passage through RNeasy columns (Qiagen, Valencia, CA). Microarray chips (Affymetrix, Santa Clara, CA) will be used to analyze changes in the expression of more than 47,000 transcripts [5].

References:

1 Thorsen J, Stokholm J, Rasmussen MA, *et al.* The Airway Microbiota Modulates Effect of Azithromycin Treatment for Episodes of Recurrent Asthma-like Symptoms in Preschool Children: A Randomized Clinical Trial. *Am J Respir Crit Care Med* 2021;**204**:149–58. doi:10.1164/rccm.202008-3226OC

2 Bisgaard H, Hermansen MN, Buchvald F, *et al.* Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;**357**:1487–95. doi:10.1056/NEJMoa052632

3 Li X, Stokholm J, Brejnrod A, *et al.* The infant gut resistome associates with E. coli, environmental exposures, gut microbiome maturity, and asthma-associated bacterial composition. *Cell Host & Microbe* Published Online First: 21 April 2021. doi:10.1016/j.chom.2021.03.017

4 Følsgaard NV, Chawes BL, Rasmussen MA, *et al.* Neonatal Cytokine Profile in the Airway Mucosal Lining Fluid Is Skewed by Maternal Atopy. *Am J Respir Crit Care Med* 2012;**185**:275–80. doi:10.1164/rccm.201108-1471OC

5 Proud D, Turner RB, Winther B, *et al.* Gene expression profiles during in vivo human rhinovirus infection: insights into the host response. *Am J Respir Crit Care Med* 2008;**178**:962–8. doi:10.1164/rccm.200805-670OC

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