SUPPLEMENT 1

This supplement contains the following items:

1. Original protocol and protocol changes for phase 1, age 0-3
2. Original statistical analysis plan and changes to the analysis plan for
    a. Phase 1, age 0-3
    b. Phase 2, age 3-6
3. Original protocol for phase 2, age 3-6.

1. Original protocol and protocol changes

Original Protocol

The following is an English translation of the original protocol in Danish.

Local Ethics Committee: H-B-2009-014; Approved: 23-02-2009
Danish Health and Medicines Authority: 2612-3959; Approved: 23-02-2009
ClinicalTrials.gov: NCT00856947
EudraCT: 2008-007871-26

Aim

To investigate whether supplementation with high-dose vitamin D during third trimester of pregnancy has a favorable effect on the development of asthma and related disorders in the offspring.

Hypothesis

High-dose vitamin D_3 supplementation during third trimester of pregnancy will reduce the risk of developing asthma in the offspring.

Background

Asthma, eczema and allergy are the most common chronic diseases among children and over the past 40 years, the incidence of these diseases has increased in industrialized countries through yet unknown factors in the environment.

Decreased levels of maternal vitamin D in pregnancy and thereby reduced fetal vitamin D levels in utero are among the early environmental exposures suspected to have an influence on the increased incidence of asthma in children.[1] Based on epidemiological studies, a high intake of vitamin D during pregnancy has been associated with protective effects on asthmatic symptoms in young children.[2,3] Preliminary results of a newer study indicates twice the risk of asthmatic symptoms in preschool children with low vitamin D levels at birth compared to children with a high level of vitamin D levels at birth.[4]

The results are consistent with several other studies, which suggest that the population in westernized countries have a reduced supply and level of vitamin D leading to an increased risk of various diseases. E.g., vitamin D levels in the fetus has been associated with the development of schizophrenia, diabetes mellitus and bone development.[5–7] Furthermore, high levels of vitamin D in adults appears to protect against a number of diseases, including bone diseases and cancer. [8–10]

The reason for these reduced levels of vitamin D may be found in the lifestyle of modern society. The majority of our vitamin D supply derives from sun exposure, and because of increasing awareness of harmful effects of sun exposure in relation to skin cancer, our supply of vitamin D has been markedly reduced. This is a recent development, which has led to the hypothesis that the current levels of vitamin D is too low according to the level for which we are genetically programmed.

Vitamin D level is however associated with and highly influenced by other factors as well. Therefore, it is necessary to conduct controlled, blinded studies on the effect of vitamin D supplementation to provide sufficient basis for future recommendations.

Method and trial procedure

The women are recruited from the $COPSAC_{2010}$ cohort; Local Ethics Committee (H-B-2008-093), Danish Data Protection Agency (2008-41-2599).

The study is a double-blinded, placebo-controlled, randomized parallel group design. 800 pregnant women will be randomized in a 1:1 ratio to intake of either high dose vitamin D supplementation or placebo according to one of the following regimes:

1) Placebo (+ guidance in recommended supplement of vitamin D (400units daily)) or

2) High dose vitamin D supplement (2400units daily) (+ guidance in recommended supplement of vitamin D (400units daily))

The regimes are administered orally as 2 tablets daily.

Blinding and randomization are carried out by the Capital Region Pharmacy and stratified according to treatment group in the fish oil intervention study (ClinicalTrials.gov: NCT00798226). This allows for equal numbers receiving high dose vitamin D supplementation in both the fish oil active group and the fish oil placebo group.

The intervention is initiated at the beginning of the third trimester (pregnancy week 24) and continued until 1st visit to the COPSAC clinic after birth at week 1-2 postpartum. At the clinical visit in pregnancy week 24, the women will be provided with the intervention treatment and interviewed about current daily vitamin D intake and history of diseases likely to influence vitamin D levels. At pregnancy week 36 adherence to the regime will be assessed by interview at the COPSAC clinic. Furthermore, the women will be instructed to return the remaining tablets at the end of the intervention for evaluation of their compliance.

At pregnancy week 24 and 1st visit after birth a blood sample will be drawn from the mother in order to measure 25-OH-vitamin D, total calcium, parathyroid-hormone (PTH) and alkaline phosphatase.

Inclusion criteria

The study population consists of healthy pregnant women and their children participating in the $COPSAC_{2010}$ cohort. Vitamin D supplements are administered during the third pregnancy trimester. The women will be included in the study independent of residence, age, race and social status during week 22-26 of pregnancy.

Exclusion criteria

Pregnant women are excluded from the trial, if they carry a disease leading to an increased risk of potential side effects from high-dose vitamin D supplementation: Endocrinologic disease in the form of calcium metabolic disorders, parathyoroidea disease, thyroid disorders or type 1 diabetes; Tuberculosis; Sarcoidosis or illness requiring chronic treatment with diuretics or heart medications, including calcium channel blockers or if they have a current intake of vitamin D supplements over the recommended dose.

Risks and disadvantages:

Known potential adverse effects of vitamin D intoxication is hypercalcemia and accompanying symptoms such as loss of appetite, nausea, vomiting, weight loss, headache, lethargy, fatigue, confusion and renal impairment. These side effects are not found by the administration of vitamin D in physiological doses. Vitamin D intoxication occurs only by the intake of very high doses of Vitamin D (4 times higher doses than administered in our study). In order to avoid administering vitamin D supplements to women with a high initial level, women with an intake above the recommended dose in the previous 6 months are excluded. Expected disadvantages related to blood sample procedures and are temporary in nature without the risk of permanent injury.

Ethical aspects

Oral vitamin D supplement has been shown to be safe and non-toxic in many randomized trials, including studies involving pregnant women. The risk of adverse effects in the pregnant woman or the fetus is suspected to be minimal. Based on the previous studies, it is expected that a large proportion of the participating women will have a daily low Vitamin D level, and therby vitamin D supplementation to these women will be a health benefit. The control group receive recommended dose of vitamin D, and ethical problems in relation to sufficient treatment of the control group is thereby not a problem.

We believe that the study as outlined above is ethically acceptable and randomized trials of vitamin D supplements are necessary for future recommendations of vitamin D intake.

Changes to the protocol

Changes to the original protocol are indicated in https://clinicaltrials.gov/ct2/show/NCT00856947

Briefly, these encompass introduction of novel assessments, including neurological development, growth, systemic immune status and airway mucosal immune status.

Reference List

1 Litonjua AA, Weiss ST. Is vitamin D deficiency to blame for the asthma epidemic? *J Allergy Clin Immunol* 2007;**120**:1031–5. doi:10.1016/j.jaci.2007.08.028

2 Devereux G, Litonjua AA, Turner SW, *et al.* Maternal vitamin D intake during pregnancy and early childhood wheezing. *Am J Clin Nutr* 2007;**85**:853–9.

3 Camargo CA, Rifas-Shiman SL, Litonjua AA, *et al.* Maternal intake of vitamin D during pregnancy and risk of recurrent wheeze in children at 3 y of age. *Am J Clin Nutr* 2007;**85**:788–95.

4 Camargo CA, Ingham T, Wickens K, *et al.* Cord-Blood 25-Hydroxyvitamin D Levels and Risk of Respiratory Infection, Wheezing, and Asthma. *Pediatrics* 2011;**127**:e180–7. doi:10.1542/peds.2010-0442

5 McGrath J, Selten J-P, Chant D. Long-term trends in sunshine duration and its association with schizophrenia birth rates and age at first registration — data from Australia and the Netherlands. *Schizophr Res* 2002;**54**:199–212. doi:10.1016/S0920-9964(01)00259-6

6 Hyppönen E, Läärä E, Reunanen A, *et al.* Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *The Lancet* 2001;**358**:1500–3. doi:10.1016/S0140-6736(01)06580-1

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8 Bischoff-Ferrari HA, Giovannucci E, Willett WC, *et al.* Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. *Am J Clin Nutr* 2006;**84**:18–28.

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2. Original statistical analysis plan and changes to the analysis plan

a) Original statistical analysis plan, phase 1, age 0-3

Outcome definitions:

Primary outcome

Persistent wheeze
Description: Age at onset of persistent wheeze in the first 3 years of life diagnosed according to a predefined algorithm of recurrent troublesome lung symptoms, response to treatment and relapse after withdrawal of treatment

Secondary outcomes

Asthma exacerbations
Description: Age at onset of severe asthma exacerbations diagnosed by predefined criteria of acute severe asthma requiring oral/high dose inhaled steroids or acute hospital contact

Eczema
Description: Age at onset of eczema diagnosed prospectively by research doctors according to predefined algorithm based upon Hanifin and Rajka criteria

Allergic sensitization
Description: Allergic sensitization at 6 and/or 18 months of age assessed by skin prick test and specific IgE in blood

Infections
Description:
Main analysis: Number of lower respiratory tract infections registered in daily diaries
Secondary analyses: Acute otitis media, number of upper respiratory tract infections, number of other infections, total number of infections

Statistical analyses:

The effect of high-dose Vitamin D_3 supplementation on age at onset of persistent wheeze, lower respiratory infections, and eczema is analyzed by Cox proportional hazards regression, where p-values correspond to Wald tests. The children are retained in the model from birth until age of diagnosis, drop out, or age at their last clinic visit before the RCT was unblinded.

The effect of Vitamin D_3 supplementation on the cross-sectional end-points asthma and allergic sensitization is analyzed by logistic regression, whereas the effect on number of wheezy episodes and upper respiratory infections is analyzed by a generalized estimating equation (GEE) Poisson regression model.

The effect on airway immunology is analyzed by calculating geometric mean ratios of each mediator in the high-dose Vitamin D_3 vs. control group and by a principal component analysis (PCA) capturing the overall immunological trends in the data and their relation to the intervention analyzed by Wilcoxon rank sum test. Initially, the mediator levels were log-transformed. Prior to the PCA the variables ware scaled to unit variance.

The primary analysis of persistent wheeze is presented crude and adjusted for sex, birth season, maternal Vitamin D level at randomization, and the n-3 LCPUFA RCT.

A significance level of 0.05 is used in all types of analyses.

Changes to the statistical analysis plan

Power calculation

A power calculation was performed based upon the available number of 587 children participating in the Vitamin D trial. The Vitamin D_3 RCT had a 65% power to detect a difference between the treatment groups (alpha=0.05, two-tailed) based on the 587 included children, an effect of 0.5 in the Vitamin D_3 supplementation group, and a 12% expected frequency of persistent wheeze in the control group.

Additional secondary endpoints:

The novel assessments introduced in the cohort resulted in additional secondary end-points:

Airway mucosal immune status
Description: Immune status measured in airway mucosal lining fluid at 4 weeks and 2 years of age (combined assessments by prinicipal component analyses for each age point)

Systemic immune status
Description:
Main analysis: Immune status at 18 months measured in stimulated whole blood as cytokine release (combined assessments by principal component analyses)
Secondary analysis: Composition of immune cell subsets in whole blood at birth and at 18 months of age

Neurological development 0-3 years
Description:
Main analysis: Cognitive development assessed at 2½ years using the cognitive part of Bayley Scales of Infant and Toddler Development, third edition
Secondary analyses: 1) Milestone development monitored prospectively by the parents using a registration form based on The Denver Development Index and WHO milestones registration (combined assessment by principal component analysis); 2) Language development assessed at 1 and 2 years of age with the Danish version of The MacArthur Bates Communicative Developmental Inventory (CDI); 3) The child´s general development (language, fine and gross motor, social and problem solving) at 3 years of age assessed with Ages and stages Questioner, third edition (ASQ-3)

Growth
Description:
Main analysis: Body composition (fat mass and bone mineral density) assessed by DEXA scan at 3 years of age
Secondary analysis: Development of BMI from birth to 3 years assesses longitudinally in the research clinic.

b) Statistical analysis plan, phase 2, age 3-6

Outcome definitions:

Primary outcome

Asthma
Description: Asthma is diagnosed according to the same predefined algorithm of recurrent troublesome lung symptoms, response to treatment and relapse after withdrawal of treatment, which was used for persistent wheeze at age 0-3.
Primary outcome in phase 2 is current asthma at age 6, which is diagnosed in children fulfilling the persistent wheeze algorithm at any point during the first 6 years of life and still needing inhaled corticosteroids at age 6 to remain well controlled.

Secondary outcomes

Lung function
Description: Age 6 mesurements of FEV1, MMEF, FEV1/FVC ratio from spirometry and sRaw from plethysmography.

Bronchial reactivity
Description: Provocative dose of methacholine leading to a 20% drop in FEV1 from baseline (PD20) at age 6.

Airway inflammation
Decsription: Fractional of exhaled nitric oxide (FeNO) at age 6.

Allergic sensitization
Description: Allergic sensitization at age 6 assessed by skin prick test and specific IgE in blood.

Allergic rhinitis

Description: Allergic sensitization combined with troublesome congestion or sneezing or runny nose upon relevant exposure to allergens at age 6.

Statistical analyses:

Primary outcome: The effect of high-dose Vitamin D_3 supplementation on the primary outcome asthma at age 6 is analyzed with logistic regression presented crude and adjusted for sex, birth season, maternal Vitamin D level at randomization, and the n-3 LCPUFA RCT.

Secondary outcome analyses: The effect of Vitamin D_3 supplementation on the cross-sectional end-points allergic sensitization and rhinitis is analyzed by logistic regression, whereas the effect of continuous outcomes (FEV1, MMEF, FEV1/FVC ratio, sRaw, FeNO and PD20) is analyzed with linear regression models. FeNO is log-transformed prior to analysis. PD20 is calculated by fitting a logistic regression function to the dose-response curves; the PD20 values are log-transformed prior to analysis.

A significance level of 0.05 is used in all types of analyses.

3. Original protocol for phase 2, age 3-6.

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The Capital Region of Denmark
Regionsgården
Kongens Vænge 2
3400 Hillerød

Gentofte 9/9/2013

Notification to The National Committee on Health Research Ethics – Additional Protocol Phase 2, 3-6y follow-up
All the information stated in this document are available for publication.

Committee

Primary Committee:	The National Committee on Health Research Ethics
Project ID:	H-B-2008-093
Notification nr.:	39915
Additional Protocol nr.:	15

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C. Project information

1. Project title:	Asthma Begins in Childhood – ABC-cohort; in publications named the COPSAC2010.
2. Changes:	We want to continue the ABC-cohort study trial with embedded DB-RCT of high-dose vitamin D and n-3 LCPUFA during pregnancy with 3 scheduled clinic visits at age 4, 5 and 6 years to follow-up on the primary outcome of the DB-RCT. The main objectives of the trial are unchanged and we still focus on asthma development as well as new methods to predict the risk of asthma, allergy, hay fever and eczema. Additionally, we focus on other relevant factors influencing the child's general health and growth and use the same tests as in earlier clinical visits. New measurements are added when the child can cooperate at the age 6 years: spirometry, bronchial reactivity to metacholine, fractional exhaled nitric oxide (FeNO) measurements and scrape samples from the nasal mucous membrane for mRNA analysis.

3. Reason for changes:	As the children have been followed during 11 scheduled clinic visits from pregnancy till 3 years of age, we have already collected a large amount of data regarding influencing factors that could affect the child in the first years of life. Evidence suggests that causes for development of asthma, allergy and eczema is to be found in early life and already in pregnancy due to a possible complex interaction between genetics and environment. A follow-up on the children is very important as not many diseases are present before 3 years of age and asthma symptoms have a great variation over time. The additional lung function measurements (bronchial reactivity and FeNO) have been used for many years in the diagnosis and follow-up of asthma in both children and adults. The nasal scrape is used in the analysis of nasal membrane mRNA, this a helpful tool to understand which genes are active in disease and can be linked to the gene analyses in the blood samples.
4. Ethical considerations:	None of the above tests are associated with any kinds of health risks. The nasal scrape can be considered slightly uncomfortable and in some cases cause nosebleed. Spirometry, plethysmography, metacholine test, FeNO measurement and nasal scrape are considered harmless and closely monitored by the doctors. Therefore, we consider the implementation of the new tests safe and without any ethical issues.
5. Is new participant information required due to the changes?	Yes

Signature of responsible investigator:

Underskrift

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Overview of clinical tests; phase 2:

Children	**4 years**	**5 years**	**6 years**	**Investigations of biologic material**
Airway and eczema diary	x	x	x	
Doctor examination			x	
Tympanometry			x	
Blood pressure			x	
Growth details	x	x	x	
Urine sample			x	Interleukins, leukotrienes and metabolic products
Blood sample			x	sIgE, immune system, epigenetics and metabolomics
Skin prick test			x	Allergy
Hair sample			x	Cotinine (nicotine product)
Faeces sample	x	x	x	Microbiological colonization
Skin swab			x	Microbiological colonization
Throat swab			x	Microbiological colonization
Nasal scrape			x	mRNA - gene expression
Activity measure			x	
DXA scan			x	Bone density
Spirometry			x	
Bodybox	x		x	
Metacholine provocation			x	
FeNO measure			x	
Child Behavior Checklist			x	Mentality and behavior
Parents				
Interview - child environment	x	x	x	
Interview - child asthma and allergy	x	x	x	