

1 **SUPPLEMENT 1**

2 This supplement contains the following items:

3 1. Original protocol and protocol changes.

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

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6 **1. Original protocol and protocol changes**

7 **Original Protocol**

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

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

13 **Aim**

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

16 **Hypothesis**

17 High-dose vitamin D₃ supplementation during third trimester of pregnancy will reduce the risk of developing asthma in
18 the offspring.

19 **Background**

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

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

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

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

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

41 **Method and trial procedure**

42 The women are recruited from the COPSAC₂₀₁₀ cohort; Local Ethics Committee (H-B-2008-093), Danish Data
43 Protection Agency (2008-41-2599).

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

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

48 2) High dose vitamin D supplement (2400units daily) (+ guidance in recommended supplement of vitamin D
49 (400units daily))

50 The regimes are administered orally as 2 tablets daily.

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

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

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

62 **Inclusion criteria**

63 The study population consists of healthy pregnant women and their children participating in the COPSAC₂₀₁₀ cohort.
64 Vitamin D supplements are administered during the third pregnancy trimester. The women will be included in the study
65 independent of residence, age, race and social status during week 22-26 of pregnancy.

66 **Exclusion criteria**

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

72 **Risks and disadvantages:**

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

80 **Ethical aspects**

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

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

89 **Changes to the protocol**

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

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

93 **Reference List**

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

117 **Original statistical analysis plan**

118 **Outcome definitions:**

119 Primary outcome

120 Persistent wheeze

121 Description: Age at onset of persistent wheeze diagnosed according to a predefined algorithm of recurrent troublesome
122 lung symptoms, response to treatment and relapse after withdrawal of treatment

123 Secondary outcomes

124 Asthma exacerbations

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

127 Eczema

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

130 Allergic sensitization

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

132 Infections

133 Description:

134 Main analysis: Number of lower respiratory tract infections registered in daily diaries

135 Secondary analyses: Acute otitis media, number of upper respiratory tract infections, number of other infections, total
136 number of infections

137 **Statistical analyses:**

138 The effect of high-dose Vitamin D₃ supplementation on age at onset of persistent wheeze, lower respiratory infections,
139 and eczema is analyzed by Cox proportional hazards regression, where p-values correspond to Wald tests. The children
140 are retained in the model from birth until age of diagnosis, drop out, or age at their last clinic visit before the RCT was
141 unblinded.

142 The effect of Vitamin D₃ supplementation on the cross-sectional end-points asthma and allergic sensitization is
143 analyzed by logistic regression, whereas the effect on number of wheezy episodes and upper respiratory infections is
144 analyzed by a generalized estimating equation (GEE) Poisson regression model.

145 The effect on airway immunology is analyzed by calculating geometric mean ratios of each mediator in the high-dose
146 Vitamin D₃ vs. control group and by a principal component analysis (PCA) capturing the overall immunological trends
147 in the data and their relation to the intervention analyzed by Wilcoxon rank sum test. Initially, the mediator levels were
148 log-transformed. Prior to the PCA the variables were scaled to unit variance.

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

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

152 **Changes to the statistical analysis plan**

153 Power calculation

154 A power calculation was performed based upon the available number of 587 children participating in the Vitamin D
155 trial. The Vitamin D₃ RCT had a 65% power to detect a difference between the treatment groups (alpha=0.05, two-
156 tailed) based on the 587 included children, an effect of 0.5 in the Vitamin D₃ supplementation group, and a 12%
157 expected frequency of persistent wheeze in the control group.

158 Additional secondary endpoints:

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

160 Airway mucosal immune status

161 Description: Immune status measured in airway mucosal lining fluid at 4 weeks and 2 years of age (combined

162 assessments by principal component analyses for each age point)

163 Systemic immune status

164 Description:

165 Main analysis: Immune status at 18 months measured in stimulated whole blood as cytokine release (combined

166 assessments by principal component analyses)

167 Secondary analysis: Composition of immune cell subsets in whole blood at birth and at 18 months of age

168 Neurological development 0-3 years

169 Description:

170 Main analysis: Cognitive development assessed at 2½ years using the cognitive part of Bayley Scales of Infant and

171 Toddler Development, third edition

172 Secondary analyses: 1) Milestone development monitored prospectively by the parents using a registration form based

173 on The Denver Development Index and WHO milestones registration (combined assessment by principal component

174 analysis); 2) Language development assessed at 1 and 2 years of age with the Danish version of The MacArthur Bates

175 Communicative Developmental Inventory (CDI); 3) The child's general development (language, fine and gross motor,

176 social and problem solving) at 3 years of age assessed with Ages and stages Questioner, third edition (ASQ-3)

177 Growth

178 Description:

179 Main analysis: Body composition (fat mass and bone mineral density) assessed by DEXA scan at 3 years of age

180 Secondary analysis: Development of BMI from birth to 3 years assessed longitudinally in the research clinic

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