The effect of vitamin C on the severity of asthma

Protocol for a subgroup analysis

VERSION 4
Oct 28, 2010

Harri Hemilä, Mohamed Al-Biltagi, Ahmed Abdul Baset, Mohammed Attia

Harri Hemilä, MD, PhD
Department of Public Health
University of Helsinki
Helsinki, Finland
harri.hemila@helsinki.fi

Mohamed Al-Biltagi, MD, PhD
Department of Paediatric
Faculty of Medicine
Tanta University
Tanta, Gharbia Governorate, Egypt
mbelrem@hotmail.com

Ahmed Abdul Baset, MD, PhD
Department of Paediatric
Faculty of Medicine
Tanta University
Tanta, Gharbia Governorate, Egypt
ahmad.baste@yahoo.com

Mohammed Attia, MD, PhD
Department of Clinical Pathology
Faculty of Medicine
Tanta University
Tanta, Gharbia Governorate, Egypt
attia17@yahoo.com.uk

Background for the subgroup analysis

In 2009, Al-Biltagi et al. published a RCT in which they administered 5 different treatments at randomly ordered phases to 60 Egyptian children, so that each participant served as his or her own control (cross-over design). One of the phases was 0.2 g/day vitamin C and another was the placebo, and these two phases are the focus of this subgroup analysis. The methods and overall results are described in Biltagi et al. 2009.

When Hemilä was preparing a Cochrane review on vitamin C and asthma, he asked Al-Biltagi to send the original data for the FEV1 and childhood asthma control test (C-AST) so that appropriate means and SDs could be calculated for the purpose of the Cochrane review.

As the effect of vitamin C on C-AST and FEV1 was statistically highly significant in the Al-Biltagi trial, Hemilä carried out a subgroup analysis to examine whether the effect of vitamin C on FEV1 would depend on baseline FEV1 level (see rationalization below). When the participants were divided into two groups by the median initial FEV1, the difference between the two groups was significant (interaction test). Because of this finding, Hemilä suggested to Al-Biltagi that a formally planned subgroup analysis of the vitamin C effects would be justified. No further subgroup analyses were carried out before this protocol was prepared (i.e. in addition to the modification of vitamin C effect on FEV1 by baseline FEV1).

Because of the multiple comparisons problem associated with subgroup analysis, Hemilä and Al-Biltagi prepared this protocol before initiating further subgroup comparisons. This protocol was revised by Hemilä and Al-Biltagi until both of them were satisfied. Hemilä started to analyze the data only after this protocol was considered final.

This protocol will be submitted as a supplementary file with the manuscript. If there will be relevant differences between this protocol and the actual subgroup analysis, the differences will be described in the manuscript; or, if there is no space in the manuscript, then the differences will be described as a new appended paragraph at the beginning of this protocol.

Basis to examine heterogeneity in the effect of vitamin C on asthma

Usually it is assumed that the effect of an intervention is uniform over the study population. This is partly caused by the need for simple assumptions for the power calculation at the planning stage. In addition, at the analysis stage, testing the overall effect is simple compared with appropriate testing of subgroup differences. Furthermore, trials testing subgroup differences would need substantially more participants compared with trials testing the uniform effect.

However, biology is complex and there is no basis to assume that all interventions have the same universal effect on all people.

In the case of vitamin C, there is strong evidence that its effect on common cold incidence is different between people under heavy acute physical stress and people of the general community [Hemilä 1996, 2007]. There is also evidence that the effect on common cold may vary between sexes, so that the effect may be greater for males [Hemilä 1997, 2008a, Constantini 2010]. Finally, there is some evidence that the effect may be greater for population groups that have low dietary intake of vitamin C [Hemilä 1997].

In the case of vitamin E, a fat-soluble antioxidant which interacts with vitamin C, the evidence of heterogeneity is much stronger still. The effect of vitamin E supplementation on common cold incidence was modified by age at the follow-up visit, smoking, and residential neighborhood [Hemilä 2006a], while its effect on pneumonia was modified by age, level of baseline smoking, age of smoking initiation, weight and vitamin C intake [Hemilä 2004, 2006b, 2008b]. Furthermore, strong evidence of second-order interaction was found between the vitamin E effect on common cold incidence and age and the level of baseline smoking [Hemilä 2006a] and between the vitamin E effect on mortality and age and vitamin C intake [Hemilä 2009].

We do not assume that heterogeneity in the effect of vitamin C on the common cold, or in the various effects of vitamin E, implies that the effect of vitamin C on asthma is heterogeneous. Nevertheless, we consider that those previous findings of heterogeneity give a sound rationalization to examine the possible heterogeneity in the vitamin C effect on asthma.

Biologic reasoning for the vitamin C and asthma relationship

If dietary vitamin C intake has an effect on airway inflammation and bronchoconstriction, we expect dose-dependency. When dietary vitamin C intake is low, then airway inflammation and bronchoconstriction may be more pronounced when compared with the state of high vitamin C intake. In converse, this means that high level of airway inflammation and bronchoconstriction might be explained partly by low vitamin C intake level. Therefore the effect of vitamin C supplementation might be most pronounced when the dietary vitamin C intake is low.

Dietary vitamin C intake was not examined in the Al-Biltagi et al. trial, and therefore subgroup analysis by dietary vitamin C intake cannot be carried out. However, this assumed dose-dependency between vitamin C and pulmonary effects also implies that the effect of vitamin C supplementation might be greater for those participants who have low FEV1 and C-AST values (low C-AST means more symptoms). Similarly, the effect of vitamin C might be greater for those who have high level of leukotrienes C4 or E4 (LTC4, LTE4) or prostaglandin D2 (PGD2)(which are bronchoconstrictors) or interleukin 1-beta (IL1B) (which is proinflammatory) [Barnes 1998, 2008].

With the evidence of sex differences in the common cold trials (see above), we assume that the effect of vitamin C on asthma might be greater on boys compared with girls.

Given that vitamin C is an antioxidant and smoking causes oxidative stress [e.g. Bruno 2005], we hypothesize that the effect of vitamin C might be greater on children who have a higher level of oxidative stress caused e.g. by parental smoking. Dampness or mold in the bedroom might cause irritation and inflammation of airways and vitamin C might have a greater effect on patients who are exposed to them.

An analysis of vitamin E effect on common cold incidence in middle-aged males found modification of vitamin E effect by the residential neighborhood: city vs. smaller neighborhoods so that the benefit of vitamin E for old males was greater in cities [Hemilä 2006a]. This might be caused by a higher level air pollution in cities or by the more frequent contacts between people in cities (transmission of viruses). We consider it possible that the effect of vitamin C on asthma might depend on the residential neighborhood, and we assume the same direction so that we expect the effect of vitamin C to be greater in cities.

The multiple comparison problem

For a single comparison, the difference between two groups is not easily explained by chance if $P<0.05$, and therefore such a finding is interesting. However, if 20 independent calculations of P-values are carried out for random variations (let us assume no real differences), on average one of the comparisons gives a "false positive" (at the level $P<0.05 = 1/20$). Therefore, in a large series of P-value calculations, one individual $P<0.05$ does not imply a real difference [see ref. Multiple testing].

In our subgroup analysis, the multiple comparison problem is caused by the large number of outcomes that were measured (n=8): FEV1, C-AST, sputum white blood cells, eosinophils, LTC4, LTE4, IL1B, PGD2, and the large number of baseline variables that were recorded (n>20), which might be used for subgroup analysis. Thus, over 160 P-values could be calculated ($>8\times20$) and therefore 8 values $P<0.05$ and 2 values $P<0.01$ would be expected simply by the multiple comparisons, if all possible combinations were calculated.

To limit the problem caused by the multiple comparisons, we restrict the number of primary outcomes and primary baseline variables to those that we consider most important from the biological point of view. When the list of the primary subgroup analyses is short, then more weight can be put on the possible positive findings.

When we have been planning this protocol for subgroup analysis, we have considered the recent recommendations (Kent 2010: http://www.trialsjournal.com/content/11/1/85):

Checklist for Reporting on Subgroup Analyses & Heterogeneity in Treatment Effects
http://www.trialsjournal.com/content/11/1/85/table/T4

Points 1 and 2 are not directly relevant in our study (intended for binary outcomes)

However, point 2 proposes that the effect should be analyzed by predicted risk, assuming that the effect may be greater for people who have high risk of the disease. Thus, our assumption that the vitamin C effect might be greater for participants with more serious asthma is in line with point 2 although we analyze a chronic condition and not incidence.

3. Any additional primary subgroup analysis should be pre-specified and limited to patient attributes with strong a priori pathophysiological or empirical justification.

• All primary subgroup comparisons must be pre-specified.
• Prespecification should include all aspects of the subgroup analysis, including threshold values for continuous or ordinal variables where these are used.
• All primary subgroup analyses must be justified based upon pathophysiological or empirical evidence that this factor modifies treatment effects.

4. Conduct and report on secondary (exploratory) subgroup analyses separately from primary subgroup comparisons.

• Secondary subgroup analyses must be reported separately from primary subgroup analyses and clearly labeled as exploratory (potential useful for hypothesis generation and informing future research, but having little or no immediate relevance to patient care).

5. All analyses conducted must be reported and statistical testing should be done using appropriate methods (such as interaction terms) and avoiding overinterpretation.

• Reporting must include results for all subgroup analyses conducted and the paper must state that primary subgroup analyses conducted were pre-specified and reported.
• Statistical comparisons should be limited to reporting for statistical significance of treatment heterogeneity between subgroups using interaction terms. (Testing for the significance of a treatment effect within a subgroup is inappropriate due to poor statistical power).
• Statistical comparisons should be corrected for the number of primary subgroup analyses performed.

In our preparation of this plan for subgroup analysis, we have also considered two thoughtful discussions of subgroup analysis [Rothwell 2005, Wang 2007].

Protocol for the subgroup analysis

Outcomes

We will use the following clinically relevant outcomes as the **primary** outcomes in our subgroup analysis:
1) FEV1
2) C-AST

FEV1 is a standard and widely used measure of pulmonary function. Outcome for FEV1 will be calculated as the percentage increment (difference between FEV1 after vit C period and FEV1 after the placebo period divided by FEV1 after the placebo). Calculating the percentage increment adjusts for baseline variation caused by the variation in the size of lungs. This use of percentage increment also means that, say, 15% increase in FEV1 is considered as important whatever the baseline FEV1 value had been.

As a measure of symptoms, C-AST is more directly important for patients than FEV1. However, symptom scales are not as universal as FEV1. Outcome for C-AST will be calculated as the difference (at the end of the vitamin C phase minus at the end of the placebo phase).

If the distribution of FEV1 change or C-AST change is skewed, we will test whether log or other transformation would make the distribution more symmetric. We will also consider using non-parametric tests with or instead of the parametric tests.

We may use the following surrogates as **secondary** outcomes in our subgroup analysis: Sputum: levels of white blood cells, eosinophils, LTC4, LTE4, IL1B, PGD2. For these outcomes we will calculate the ratio or the percentage increment (after vit C vs. after placebo).

Baseline variables for the subgroup divisions

See the Appendix for the baseline variable distributions.

We will carry out the **primary** subgroup analysis by:

1) FEV1 (as FEV1/FVC)
In the division of participants to subgroups by FEV1, we will use the FEV1/FVC ratio that was measured at baseline of the study, before the treatment periods were started, because the ratio FEV1/FVC adjusts for the size of the lungs.

2) Asthma control test (C-AST)
In our interaction test, we will cut the continuous variables (FEV1 and C-AST) close to the median. If there is significant interaction, we will examine the relationship in more detail.

3) Sex (38 males, 22 females)

4) Paternal smoking: Never (n=21), Ex-smoker (n=17), Occasional (n=9), Daily smoker (n=13)
In the test of interaction, we will compare Never (n=21) vs. Occasional+Daily (n=22).
We will present the findings for the subgroup of Ex-smokers, but we will not use that in our interaction test. We do not know when the fathers had stopped smoking. If father's smoking modifies the vitamin C effect, we assume that Ex-smokers would be closer to Never smokers. However, it is also possible that there are long term harms caused by exposure to father's smoking so that the children of ex-smokers might also be closer to Current smokers. Therefore, the

comparison between Never and Current (=Occasional+Daily) is most unambiguous in our analysis and Ex-smokers are not included in the interaction test.

5) Dampness or mold in the bedroom: Never: no exposure (n=25), Only Current: during the past 1 months (n=20), Only Early: before 1 year age (n= 14), both Early and Current exposure (n=1)
In the test of interaction, we will compare Never (n=25) vs. Current (n=20+1).
We will present the findings for the subgroup Early, but we will not use that in our interaction test. It is possible that early age exposure leads to long term harms so that they might modify the effect of vitamin C. The comparison between Never and Current is most unambiguous in the interaction test.

6) Residential neighborhood: Urban (n=34) vs Rural (n=26).

We may carry out **secondary** subgroup analysis by:
Age, weight, height, BMI, nasal allergy, medications for asthma and allergy, area of residence, parents education, paternal asthma, child use of extra table salt, sputum: white blood cells, eosinophils, LTC4, LTE4, IL1B, PGD2.

Primary subgroup analyses

Based on the above description, our pre-planned primary subgroup analysis will have 2 outcomes and 6 subgroup divisions, which means that 12 P-values are calculated for the interactions between the vitamin C effect and the subgroups.

The Bonferroni approach [see ref. Bonferroni correction] suggests that, when 12 P-values are calculated, then P=0.0042 (=0.05/12) should be used as the limit below which a finding cannot easily be explained by chance. We do not set up such a strict limit, because there is no real difference between slightly smaller and slightly higher P-values, so that dichotomizing by a fixed P-level is arbitrary and misleading. Nevertheless, if only one individual P-value is small, it must be substantially smaller than the conventional P=0.05 to be considered as an indication of real difference.

Another way to approach the multiple comparison problem is to calculate the expected number of P<0.05 values for a set of 12 comparisons. Assuming random variation, the probability that in a set of 12 comparisons there are exactly two values P<0.05 is 10%, but the probability that there are three or more values P<0.05 is 2%. Thus, one or two P<0.05 level differences in a set of 12 calculations can easily be explained by random variation, but 3 or more cannot. In our consideration of the number of values P<0.05 we will ignore the subgroup analysis that was already done (baseline FEV1 level and FEV1 change). Thus, there need to be 3 or more P<0.05 among the remaining 11 comparisons, before we consider that the number of P<0.05 findings is higher than expected.

All 12 primary analyses will be reported in two tables, so that one table is for each primary outcome.

Our hypothesis is in one direction. We hypothesize that the effect of vitamin C is greater for those asthma patients who have more severe symptoms. In that respect the 1-tailed P-values for the interaction test would be justified. However, to avoid confusion for ordinary readers, we will present the 2-tailed P-values.

Secondary subgroup analyses

By secondary subgroup analyses we mean the use of
a) the secondary outcomes or
b) the secondary baseline variables.

We do not formulate exact plans for exploring secondary subgroup differences, because their relevance depends on the findings of the primary analyses.

If there is unambiguous modification of vitamin C effects on one of the primary outcomes, then the secondary outcomes may be interesting from the mechanistic point of view. However, if there is no modification of vitamin C effect on either of the primary outcomes, then the possible modification of the surrogate outcomes is of minimal interest.

Similarly, if the primary baseline variables show strong evidence of interaction with vitamin C effect, then it may be reasonable to explore the modification of vitamin C effect by the secondary baseline variables (e.g. baseline level of the inflammatory markers). However, if none of the primary baseline variables modifies the vitamin C effect, then the secondary variables are of minimal interest.

For such reasons, we do not fix exact plans for the possible secondary subgroup analyses. In our manuscript we will report the number of secondary analyses that we carried out, even if we might not show them in a separate table (e.g. all findings "negative" will be stated if that is the reason for not presenting them in a table), so that reporting what was done will be independent of what was found.

Second-order interactions

Given the earlier evidence that there may be complex modifications of vitamin E effects so that several factors simultaneously determine the effect of vitamin E supplementation (see above), we consider that it may be fruitful to explore second-order interactions of the vitamin C effect on asthma. However, the relevance of exploring the second-order interactions depends on the findings on the first-order interactions.

The second-order interactions cannot be preplanned in the same sense as the primary subgroup analyses. Examination of second-order interactions is exploratory and suitable cut-off levels may be selected by viewing the data. Therefore, the P-values of the second order interactions are not as easy to interpret as the P-values of the pre-planned subgroup analysis, because several choices need to be done after the first order interaction results are seen.

If we carry out second-order interaction test, we will calculate the P-value for testing heterogeneity over all the subgroups (e.g. 2x2 groups means a test with 3 df to allow each of the four subgroups their own vitamin C effect) and for the interaction terms (e.g. 2x2 groups means a test with 1 df).

Statistical calculations

The data will be analyzed primarily by using the R program package (http://www.r-project.org/).

References

Barnes PJ, Chung KF, Page CP. Inflammatory mediators of asthma: an update. Pharmacol Rev 1998;50:515-96.
http://pharmrev.aspetjournals.org/content/50/4/515

Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. J Clin Invest 2008;118:3546-56.
http://www.jci.org/articles/view/36130

Bonferroni correction, e.g.
http://en.wikipedia.org/wiki/Bonferroni_correction

Bruno RS, Ramakrishnan R, Montine TJ, Bray TM, Traber MG. alpha-Tocopherol disappearance is faster in cigarette smokers and is inversely related to their ascorbic acid status. Am J Clin Nutr. 2005;81:95-103.
http://www.ajcn.org/cgi/content/abstract/81/1/95

Biltagi MA, Baset AA, Bassiouny M, Kasrawi MA, Attia M. Omega-3 fatty acids, vitamin C and Zn supplementation in asthmatic children: a randomized self-controlled study. Acta Paediatr. 2009;98:737-42.
http://dx.doi.org/10.1111/j.1651-2227.2008.01213.x

Constantini NW, Dubnov-Raz G, Eyal BB, Berry EM, Cohen AH, Hemilä H. The effect of vitamin C on upper respiratory infections in adolescent swimmers: a randomized trial. Eur J Pediatr. 2010 Aug 6. [Epub ahead of print]
http://dx.doi.org/10.1007/s00431-010-1270-z

Hemilä H. Vitamin C and common cold incidence: a review of studies with subjects under heavy physical stress. Int J Sports Med 1996;17:379-383

Hemilä H. Vitamin C intake and susceptibility to the common cold. Br J Nutr. 1997;77:59-72.
http://dx.doi.org/10.1017/S0007114500002889

Hemilä H, Virtamo J, Albanes D, Kaprio J. Vitamin E and beta-carotene supplementation and hospital-treated pneumonia incidence in male smokers. Chest 2004;125:557-65.
http://dx.doi.org/10.1378/chest.125.2.557

Hemilä H, Virtamo J, Albanes D, Kaprio J. The effect of vitamin E on common cold incidence is modified by age, smoking and residential neighborhood. J Am Coll Nutr. 2006a;25:332-9.
http://www.jacn.org/cgi/content/abstract/25/4/332

Hemilä H. Do vitamins C and E affect respiratory infections? PhD Thesis. Helsinki, Finland: University of Helsinki 2006b:56-57.
http://ethesis.helsinki.fi/julkaisut/laa/kansa/vk/hemila/

Hemilä H, Chalker E, Douglas RM. Vitamin C for preventing and treating the common cold. Cochrane Database Syst Rev. 2007;(3):CD000980.
http://dx.doi.org/10.1002/14651858.CD000980.pub3

Hemilä H. Vitamin C and sex differences in respiratory tract infections. Respir Med. 2008a;102:625-6.
http://dx.doi.org/10.1016/j.rmed.2007.12.011

Hemilä H, Kaprio J. Vitamin E supplementation and pneumonia risk in males who initiated smoking at an early age: effect modification by body weight and dietary vitamin C. Nutr J. 2008b;7:33.
http://dx.doi.org/10.1186/1475-2891-7-33

Hemilä H, Kaprio J. Modification of the effect of vitamin E supplementation on the mortality of male smokers by age and dietary vitamin C. Am J Epidemiol. 2009;169:946-53.
http://dx.doi.org/10.1093/aje/kwn413

Kent DM, Rothwell PM, Ioannidis JP, Altman DG, Hayward RA. Assessing and reporting heterogeneity in treatment effects in clinical trials: a proposal. Trials 2010;11:85.
http://www.trialsjournal.com/content/11/1/85

Multiple testing, e.g.
http://en.wikipedia.org/wiki/Multiple_testing

Rothwell PM. Treating individuals. Subgroup analysis in randomised controlled trials: importance, indications, and interpretation. Lancet 2005;365:176-86
http://dx.doi.org/10.1016/S0140-6736(05)17709-5

Wang R, Lagakos SW, Ware JH, Hunter DJ, Drazen JM. Statistics in medicine--reporting of subgroup analyses in clinical trials. N Engl J Med 2007;357:2189-94.
http://dx.doi.org/10.1056/NEJMsr077003

Appendix: baseline distributions

Age (yr)	
Mean	8.4
SD	1.0
Weight(kg)	
Mean	29
SD	4
Height (cm)	
Mean	129
SD	6
Gender	
Male	38
Female	22
Mother's education level	
Less than secondary	1
Secondary	16
High school	27
College or higher	16
Father's education level	
Less than secondary	2
Secondary	20
High school	29
College or higher	9
Mother's smoking habit	
None	60
Ex smoker	0
Occasional	0
Daily	0
Father's smoking habit	
None	21
Ex smoker	17
Occasional	9
Daily	13
Dampness	
Never	25
Current	20
Early	14
Early+Current	1
Residential neighborhood	
Urban	34
Rural	26

Dampness exposure

We used four indicators of exposure defined from the answers to following structured questions at the baseline:

• Mold odor: “Have you perceived mold odor in your dwellings?” (no; yes, during the past 12 months; yes, only earlier).
• Visible mold: “Have your ever had visible mold in your dwelling. (no; yes, during the past 12 months; yes, only earlier).
• Moisture: “Have you ever had wet spots in the ceilings, floors or walls of the occupied rooms in your dwelling?” (no; yes, during the past 12 months; yes, only earlier).
• Water damage: “Have you ever had water damage in your dwelling?” (no; yes, during the past 12 months; yes, only earlier). (no; yes, during the past 12 months; yes, only earlier).
• Any exposure indicator: Presence of any of the four exposure indicator.

The child is given score of
Never: No exposure
Only current: during the past 12 months
Only early : before 1 year
Both current and early

This question was adopted from:

Jaakkola JJK, Hwang BF, Jaakkola N. Home Dampness and Molds, Parental Atopy, and Asthma in Childhood: A Six-Year Population-Based Cohort Study. Environ Health Perspect 2005;113:357-361.
Simoni M, Lombardi E, Berti G, et al. Mould/dampness exposure at home is associated with respiratory disorders in Italian children and adolescents: the SIDRIA-2 Study. Occup Environ Med 2005;62:616-622.

Salt intake

A food frequency questionnaire designed specifically to assess the normal intake of salt in the diet of the child was administered to a parent (almost always the mother). Food items high in sodium that are frequently consumed were grouped into nine food groupings; portion sizes were not indicated. Parents were also asked about their child's preference for salty foods. Parents chose from four frequency categories for each of the nine food groups their child might consume, and a score was obtained from the sum of the items multiplied by the frequency of each item (maximum value 27 =3×9).
The salt preference score obtained by asking the question: "Does your child like salty foods?" (very much, 2; somewhat, 1; not at all, 0) was then added to obtain the overall salt intake score (maximum value 29 =27+2).

Salty FOOD FREQUENCY QUESTIONNAIRE
Below is a list of foods your child may eat.
Please indicate how often your child eats each of the foods.

	Most days	3-5 times times/week	1-2 times times/week	Less than once/ week
Points	**3**	**2**	**1**	**0**
Cheese slices				
Bacon or salami or bologna, etc				
Potato chips, peanuts, pretzels, corn chips, etc				
Salted crackers				
Canned or packaged soup				
Kraft dinner, lasagna				
Frozen TV dinners, pot pies, etc				
Dill pickles				
Ketchup, soy sauce				

Does your child like salty foods?
Very much 2
- Somewhat 1
- Not at all 0

Adopted from:
Demissie K, Ernst P, Donald K G, Joseph L. Usual dietary salt intake and asthma in children: a case-control study. Thorax 1996;51:59-63.