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Ana Rita Martins Ferreira da Rocha

Proteína catiónica eosinofílica correlaciona-se com o número de eosinófilos no esputo induzido em nadadores de alta competição

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Dra. Mariana Valente Farraia

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Medicina Básica

TÍTULO DISSERTAÇÃO/~~MONOGRAFIA~~ (riscar o que não interessa)

Eosinophil cationic protein (ECP) correlates with eosinophil cell counts in the induced sputum of elite swimmers

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CHAVE MÓVEL

Dedicatória

À minha família e aos meus amigos,

por todo o apoio incondicional ao longo destes 6 anos.

Eosinophil cationic protein (ECP) correlates with eosinophil cell counts in the induced sputum of elite swimmers

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Eosinophil cationic protein (ECP) correlates with eosinophil cell counts in the induced sputum of elite swimmers

ABSTRACT

Introduction: Swimming practice has been associated with eosinophilic inflammation, however the underlaying mechanisms are not fully understood. The eosinophil cationic protein in induced sputum may be used as a potential biomarker to assess airway eosinophilic inflammation among elite swimmers. The objective of this study is to characterize eosinophil cationic protein levels in sputum supernatant in elite swimmers and evaluate eosinophil cationic protein as an eosinophilic inflammatory marker.

Material and Methods: Elite swimmers annually screened in our department (n=27) were invited to participate in this cross-sectional study. Swimmers who agreed to participate (n=24, 46% girls) performed lung function and skin-prick tests. Induced sputum was also collected and analyzed for differential cell counts and eosinophil cationic protein measurements in sputum supernatant (ImmunoCAP™ 100, ECP, Thermo Fisher Scientific, Uppsala, Sweden).

Results: The median eosinophil cationic protein level was 15.60 µg/L (6.02-38.75 µg/L) and higher levels were found among boys (27.90 (11.20-46.30) µg/L *vs* 6.65 (2.82-22.80) µg/L, p=0.02). In addition, eosinophil cationic protein levels in the sputum supernatant were positively correlated with eosinophil cell counts in the induced sputum (r = 0.583, p = 0.08).

Conclusions: Eosinophil cationic protein levels correlated positively with eosinophil counts in the induced sputum in elite swimmers. The measurement of eosinophil cationic protein in sputum supernatant may be a useful marker to assess and manage eosinophilic inflammatory changes in the airways of elite swimmers.

INTRODUCTION

Swimming practice has been associated with an increased risk of airway disfunction, even in athletes with no prior history of respiratory disorders.[1, 2] Cumulative life-time exposure to chlorine by-products derived from swimming pool disinfectants can cause airway irritation, epithelial damage, airway inflammation, and pulmonary function decline.[3] The high ventilation rate during swimming may determine the quantity of inhaled chlorine by-products; elite swimmers, who spend many hours in pools, are likely to be more affected by those products.[4] In fact, eosinophilic airway inflammation has been reported after long-term exposure to the pool environment in elite swimmers, but not in recreational swimmers.[5] Previous studies reported airway vascular permeability in elite swimmers similar to individuals with asthma; thus, swimming practice may promote airway inflammation by enhancing local vascular permeability.[6] Moreover, the prevalence of atopy is higher in swimmers who are repeatedly exposed to chlorine by-products. The epithelial damage caused by swimming pools environment may facilitate mucosal penetration of allergens inducing allergic sensitization. [7-9]

The induced sputum of elite swimmers has increased eosinophil and neutrophil counts compared to healthy controls.[5, 10-12] Eosinophils contain granule proteins like eosinophil cationic protein (ECP) that has proinflammatory effects and can be released when eosinophils reach the local tissue site of inflammation.[13, 14] Increased ECP levels and eosinophils have been associated with atopic diseases, such as asthma and atopic dermatitis.[15] ECP can be found in many body fluids, including serum, sputum, and bronchoalveolar lavage fluid, and may be a potential biomarker of airway eosinophilic inflammation.[16] Sputum supernatant is a fluid obtained during sputum collection and analysis, which may be useful to study and quantify several biomarkers. Previous studies

have shown its utility to assess and monitor lower airway inflammation among individuals with asthma.[17, 18] The use of sputum allows a direct and representative sample of the local inflammation, and therefore a more accurate measure to identify the presence, type, and severity of airway inflammation.

The combined effects of chronic endurance exercise and repeated exposure to chlorine by-products may cause structural and functional changes, including airway remodeling and increase of inflammatory mediators.[1] Despite the efforts of previous studies on elite swimmers, the mechanisms of airway disorders in this population are not fully understood. We believe that by characterizing inflammatory markers, we can better understand the mechanisms underlying airway changes reported in competitive swimmers. In this study, we aim to characterize the ECP levels in the sputum supernatant among elite swimmers. Furthermore, we hypothesize that ECP levels may be used as a marker of eosinophil counts in the induced sputum of elite swimmers.

MATERIAL AND METHODS

Study Design

The participants included in this cross-sectional study have been previously described.[19] Briefly, from the 27 elite swimmers screened in our department, 24 agreed to participate in this study. Swimmers eligible for this study aged ≥14 years old, trained at least ten hours per week, and were free from respiratory infection in the last 3 weeks. The screening visits occurred at the beginning of the training season, during the summer. In these visits, swimmers were asked to withhold anti-asthmatic and/or anti-allergic medication and performed a specific evaluation to collect induced sputum before the

training session. Written informed consent and ethical commission approval were obtained previously to any study procedure.

Study procedures

Lung function. Spirometry was performed according the European Respiratory Society guidelines [20], and reversibility was assessed 15 min after 400 ug of inhaled salbutamol. Clinically significant reversibility was defined as a ≥12% and 200 mL increase in the forced expiratory volume in the first second of expiration (FEV1) after inhalation of salbutamol.

Skin prick tests. Skin prick tests were performed in accordance with the international guidelines [21] using a standard battery of commercial extracts for common aeroallergens (Leti®, Madrid, Spain). Atopy was defined by the presence of a positive test to at least one of the allergens.

Collection and analysis of induced sputum. Sputum collection and analysis were performed as previously described.[22] Briefly, sputum induction was performed using an inhalation of hypertonic saline (4.5%) through a mouthpiece connected to an ultrasonic nebulizer (OMRON NE-U17; Omron Healthcare Europe, Netherlands) [23]. Sputum was then sampled and treated with dithiothreitol (Sputolysin; Calbiochem Corporation, USA). The suspension was centrifuged; the cell pellet was resuspended while supernatant was collected and stored. Cytospins were prepared and stained using May-Grunwald/Giemsa. Differential cell counts were made by counting a minimum of 500 nonsquamous cells. The lower respiratory origin was confirmed by the presence of macrophages and bronchial epithelial cells. Normal values for sputum differential cell percentages were based on published healthy subjects counts.[24-27] These measurements were done blindly to the clinical characteristics of the subjects.

Quantification of molecular markers in induced sputum supernatant. ECP in sputum supernatant was measured using ImmunoCAP™ 100, ECP, Thermo Fisher Scientific, Uppsala, Sweden. The detection limit was 0,5 µg/L.

Participants

The median age of participants was 17 years old. The prevalence of asthma was 21% (n=5) according to self-reporting medical diagnosis and 4% (n=1) had atopy (Table 1). Differential cell counts were performed only in 10 participants (3 girls and 7 boys). Participants with asthma showed a poorer lung function, specifically, a lower FEV1 and FEF25-75 percentages (Table S1). Participants with asthma practiced swimming for longer [median (25^{th}–75^{th} percentile): 12 (10-12) *vs* 9 (7-11) years, p=0.05]. Although not significant, eosinophil cell counts percentages were higher in swimmers with asthma [median (25^{th} –75^{th} percentile): 0.5 (0.1-2.1) vs 0.2 (0.0-0.2), p= 0.61] (Table S1).

Table 1 - Demographic characteristics of swimmers.

Characteristics	**Total (n = 24)**	Girls (n = 11)	Boys (n =13)	**p-value**
Age, years *	17.00 (16.00-20.50)	17.00 (14.00-19.00)	17.00 (16.00-21.00)	0.13
Years of competition *	10.00 (7.00-11.50)	9.00 (7.00-10.00)	10.00 (7.00-12.00)	0.33
Training hours per week	18.00 (16.00-20.00)	18.00 (17.00-19.00)	18.00 (14.00-20.00)	0.87
Asthma, n (%)	5 (21)	2 (18)	3 (23)	0.77
Atopy, n (%)	1 (4)	1 (9)	0	-
FVC, % *	114.50 (107.00-122.00)	115.00 (107.00-122.00)	114.00 (110.00-124.00)	0.65
FEV1, % *	115.50 (105.00-124.00)	111.00 (105.00-117.00)	119.00 (104.00-126.00)	0.27
FEV1/FVC, % *	85.20 (81.70-93.10)	84.30 (82.50-93.70)	86.15 (80.70-93.05)	0.65
FEF25-75, % *	112.00 (81.00-128.00)	98.00 (83.50-114.00)	117.00 (80.00-128.00)	0.37
FEV1 increase after BD, % *	4.50 (1.00-7.00)	5.00 (1.00-7.00)	3.00 (0.50-6.00)	0.41

*Data are presented as median (25^{th} percentile – 75^{th} percentile); FVC: forced vital capacity; FEV1: forced expiratory volume in the first second of expiration; FEF25-75: forced expiratory flow middle portion of FVC; BD: bronchodilation.

Statistical Analysis

Categorical variables were summarized as absolute numbers and percentage, and continuous variables were expressed as median (25^{th} percentile – 75^{th} percentile). Given the small sample size, nonparametric tests were used to assess differences by sex and asthma diagnosis. Mann-Whitney U-test was used for continuous variables and Chi-square tests for categorical variables. The correlation between ECP levels and eosinophils in induced sputum was assessed using the Spearman rank correlation. Statistical significance was set as p-values less than 0.05. Statistical analyses were performed using SPSS 26.0 (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, USA: IBM Corp).

RESULTS

The median ECP level was 15.60 µg/L (6.02 - 38.75 µg/L) and higher levels were found in boys (27.90 (11.20-46.30) µg/L *vs* 6.65 (2.82-22.80) µg/L, p=0.02). The median (25^{th} – 75^{th} percentile) percentages of eosinophils and neutrophils in the induced sputum were 0.18% (0 - 0.83%) and 1.15% (0.32 - 13.90%), respectively. Although no statistically significant differences were found, the median levels of eosinophils and neutrophils were higher among boys. A moderate correlation was observed between ECP levels in the sputum supernatant and eosinophils in induced sputum ($r = 0.583$, $p = 0.08$). This correlation was significant among boys ($r = 0.857$, $p = 0.01$) (Table 2). Additionally, there were no differences in the ECP levels between swimmers with asthma and healthy swimmers (Table S1).

Table 2 - Measurements of inflammatory markers.

	Total (n = 24)	**Girls (n = 11)**	**Boys (n =13)**	***p*-value**
IS neutrophils, % *	1.15 (0.32-13.90)	0.32 (0.32-2.11)	1.47 (0.45-19.30)	0.30
IS epithelial cells, % *	47.98 (18.10-51.40)	50.96 (11.81-50.96)	45.00 (18.10-57.60)	0.73
IS eosinophils, % *	0.18 (0.00-0.83)	0.00 (0.00-0.17)	0.19 (0.16-1.30)	0.08
ECP, µg/l **	15.60 (6.02-38.75)	6.65 (2.82-22.80)	27.90 (11.20-46.30)	0.02
ECP*IS eosinophils	0.58 (p = 0.08)	***	0.86 (p = 0.01)	

Data are presented as median (25^{th}– 75^{th} percentile); *IS differential cell counts were performed only in 10 participants (3 girls and 7 boys); **ECP levels were measured in sputum supernatant; ***The sample size among girls group (n = 3) was too low to perform the correlation coefficient; IS: induced sputum; ECP: eosinophilic cationic protein.

DISCUSSION

In this study, we characterize ECP levels in sputum supernatant among elite swimmers. Our results suggest that ECP may be used as a marker of eosinophilic granulocyte activation. Moreover, we found a positive correlation between ECP levels in the sputum supernatant and eosinophil cell counts in the induced sputum of elite swimmers, being significant among boys.

Our study has some limitations. First, the cross-sectional study design did not allow for monitoring the evolution of measurements. Second, variability in the execution or in the selected method for processing sputum and measuring ECP should be considered when comparing with previous studies. Third, the number of athletes assessed was small and induced sputum was performed only in a subgroup of participants, which may be explained by the complexity of collecting induced sputum with nebulized saline in non-productive subjects.[27] This technical difficulty may also explain the number of differential cell counts observed in our study. Nevertheless, sputum induction is a noninvasive, reliable, and safe method when assessing airway inflammation.[17, 28] Although sputum eosinophilic counts are a direct marker of airway inflammation, the analysis is time-consuming and technically difficult, limiting the number of individuals that can be examined simultaneously.[18, 29]

Therefore, the measurement of ECP levels in sputum supernatant to assess inflammatory response occurring in the airways of athletes might be a useful marker. Clinically, this method has the potential to be used as an evaluation tool to optimize the management, screening and prevention of airway disorders in elite swimmers. In addition, a low within-subject variability in ECP measurements has been previously reported, supporting the repeatability of this method.[24]

Similar results have been previously reported in patients with known eosinophilic airway inflammatory diseases, such as asthma, chronic obstructive disease, and cystic fibrosis.[25, 30, 31] ECP levels in our swimmers are in line with results reported for patients with asthma and higher than those found for healthy controls, considering previous studies using a similar method to measure ECP.[26, 28] Our results suggest that the mechanism behind the increment in ECP levels in elite swimmers may be independent of the diagnosis of asthma, supporting that the exposure to the swimming pool environment may play a role in these changes. In line with this finding, we also observed higher levels of ECP among boys, who had more years of competition and training hours per week, suggesting that the longer exposure to swimming pool environment, may be related to increased levels of ECP. Previous results in elite swimmers have also reported the adverse health effects from prolonged and continuous exposure to the swimming pool environment, also showing the role of irritant mechanism on airway inflammation.[2, 32] Accordingly, ECP characterization using sputum supernatant may be a simple and useful method for assessing and monitoring eosinophilic airway inflammation in elite athletes.

A 5-year prospective study reported worsening or development of eosinophilic airway inflammation among highly trained swimmers who continued their sports

career.[33] Repeated exposures to the swimming pool environment during training may partially explain this finding due chlorine and its by-products, which may irritate the airways by oxidative stress. Skrgat, Korosec [(34)] have also shown the occurrence of oxidative stress in swimmers during high intensity training period. Another study reported an enhanced migration of blood eosinophils to airway tissues during strenuous endurance exercise, leading to an increased degranulation of eosinophils and release of ECP in healthy mountaineers. [35] This finding is supported by reports of eosinophil granulocyte activation and ECP elevation during exercise that might be due to the activation of the complement system during exercise. [36] Likewise, this mechanism may also explain an increment of ECP levels in competitive swimmers.

Exercise induces hyperventilation that can increase the osmolarity of the airway surface layer by dehydration of periciliary fluid. Via an osmotic challenge to the bronchial epithelium, hyperosmolality may indirectly trigger the release of ECP from eosinophils.[10, 35, 37] Repeated bouts of exercise may lead to frequent episodes of ECP peaks increasing the risk of developing exercise-induced and allergic asthma in elite swimmers. This finding may partly explain the propensity of endurance athletes, including swimmers, for developing eosinophilic airway inflammation. Remodeling of the airway epithelium to a secretory phenotype has been previously implicated in the development of exercise-induced bronchoconstriction as a response to injury. This may be caused by the stimulation of mucin secretion during hyperpnea to counteract the drying of the protective lining of the airways and possibly increase the provision of water. Additionally, increased airway movements and pressure changes during intense breathing could stimulate epithelial cells to release cytokines. [7] This evidence indicates that eosinophilic activation and ECP release may play a role in the pathogenesis of the

airway dysfunction in elite swimmers. To support this hypothesis, ECP has been also implicated in asthma as a stimulator of airway mucus secretion.[14, 16] Our results are in line with a previous study that reported increased percentage of bronchial epithelial cells in swimmers.[12] In six non-elite outdoor swimmers, Bonsignore, Morici [(10)] observed higher baseline differential neutrophil counts in sputum when compared to controls, suggesting that chronic endurance exercise may play a role in the pathogenesis of airway neutrophilia. However, eosinophils were not elevated, suggesting that airway mast cells and eosinophilic infiltration, previously reported in swimmers [7], might be related to chronic exposure to irritants during indoor training.

However, other studies in asthma reported a weak or non-significant correlation between supernatant ECP levels and percentage of eosinophil counts.[18, 38] These contradicting results may be explained by the activation degree of eosinophils. When highly activated by specific and/or increased degranulation agents, eosinophils may release large amounts of ECP. Therefore, ECP measured in supernatant reflects the number of activated eosinophils present in sputum, which does not necessarily correlates with sputum eosinophil counts. [18] Other explanation may be related to the inclusion of diseased individuals, the total cell counts may be increased due to factors related to the disease leading to a non-specific eosinophil degranulation and a lower percentage of eosinophils, despite an increment in the absolute number of eosinophils. In fact, it has been demonstrated that the inclusion of subjects with asthma exacerbation may lead to an attenuation in the proportion of eosinophils, because there might be eosinophil lysis with release of free granules. [39]

CONCLUSION

In conclusion, the examination of ECP levels in induced sputum may be useful to examine and follow inflammatory and remodeling changes in the airways among elite swimmers. Further studies in larger populations are needed to confirm if ECP measurements in sputum supernatant is a reproducible and clinically valid method to assess airway dysfunction in this population.

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CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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PRESENTATION

None.

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SUPPLEMENTARY FILES

Table S1 Swimmers characteristics for the asthmatics and non-asthmatics groups

Characteristics	**Non-Asthmatics (n = 19)**	**Asthmatics (n =5)**	***p*-value**
Age, years *	17.0 (15.0-19.0)	21.0 (18.0-21.0)	0.04
Years of competition *	9.0 (7.0-11.0)	12.0 (10.0-12.0)	0.05
ECP	10.2 (3.5-36.2)	29.7 (11.2-86.2)	0.18
Atopy, n (%)	0	1 (20)	-
FVC, % *	116.0 (110.0-122.0)	110.0 (105.0-111.0)	0.22
FEV1, % *	117.0 (111.0-124.0)	103.0 (94.0-105.0)	0.04
FEV1/FVC, % *	91.0 (82.2-93.7)	83.8 (78.0-84.3)	0.09
FEF25-75, % *	113.0 (94.0-128.0)	78.0 (75.0-86.0)	0.04
FEV1 increase after BD, % *	4.5 (1.0-6.0)	2.0 (1.0-7.0)	0.91
IS eosinophils, % *‡	0.2 (0.0-0.2)	0.5 (0.1-2.1)	0.61
IS neutrophils, % *‡	0.9 (0.3-19.3)	1.5 (0.6-8.0)	0.76
IS epithelial cells, % *‡	48.0 (23.5-51.0)	31.6 (7.6-54.5)	0.76

*Data are presented as median (25th percentile – 75th percentile). ‡IS differential cell counts were performed only in 10 swimmers (4 asthmatics)

FVC: forced vital capacity; FEV1: forced expiratory volume in the first second of expiration; FEF25-75: forced expiratory flow middle portion of FVC; BD: bronchodilation; IS: induced sputum.

Anexo 1

Normas de Publicação da Revista *Porto Biomedical Journal*

Instructions for Authors

Note: These instructions comply with those formulated by the International Committee of Medical Journal Editors (ICMJE). For further details, authors should consult the following article: International Committee of Medical Journal Editors. “Uniform Requirements for Manuscripts Submitted to Biomedical Journals” *New Engl J Med* 1997, **336**:309–315. The complete document appears at http://www.icmje.org. Manuscripts that do not comply with these Instructions cannot be considered for publication and will be sent back to the authors.

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The paragraph could read, for example:

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4) Be accompanied by a short summary that encapsulates the report's findings for a clinically oriented audience (see above).

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6) Close with the author's name(s), academic degree(s), institutions(s), and location(s).

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Page 1:

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Authors. The full first name, middle initials, and family name of each author, as well as the name(s) of the department(s) and institution(s) to which the work should be attributed.

Address for Correspondence. A current email and full mailing address for the corresponding author must be provided.

Page 2:

Abstract. Original articles should include a structured abstract of no more than 300 words using the following headings: Background; Methods; Results; and Conclusions. They should briefly describe, respectively, the problem being addressed in the study, how the study was performed, the salient results, and what the authors conclude from the results. Conventional non-systematic, reviews should include an unstructured abstract of no more than 250 words.

Main Body: *Introduction*. The introduction contains a statement of the purpose of the work, the problem that stimulated it, and a brief summary of relevant published investigations.

Methods. Avoid detailed description of previously published methods and cite the appropriate reference. Include appropriate ethical and statistical information.

Results. The results should be concise, avoiding redundant tables and figures illustrating the same data.

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Anexo 2

Reporting Guidelines - *STROBE Statement*

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	3, 4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	4, 5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5, 6
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5, 6
Bias	9	Describe any efforts to address potential sources of bias	6
Study size	10	Explain how the study size was arrived at	4
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5, 6
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	6, 7
		(*b*) Describe any methods used to examine subgroups and interactions	6
		(*c*) Explain how missing data were addressed	6
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	7
		(*e*) Describe any sensitivity analyses	NA[1]
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	NA[2]
		(b) Give reasons for non-participation at each stage	NA[2]
		(c) Consider use of a flow diagram	NA[2]
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	NA[3]
		(b) Indicate number of participants with missing data for each variable of interest	7, 8
Outcome data	15*	Report numbers of outcome events or summary measures	7, 8
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	8

		(*b*) Report category boundaries when continuous variables were categorized	NA[4]
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA[5]
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	16
Discussion			
Key results	18	Summarise key results with reference to study objectives	8
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	8
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	9, 10, 11
Generalisability	21	Discuss the generalisability (external validity) of the study results	12
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	12

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

NA[1] – Not applicable, because we only used one method for analysis.
NA[2] – Not applicable, because all participants were included in our analysis.
NA[3] - Participants characteristics were previously described in Table 1.
NA[4] – Not applicable, because we didn't categorize our results based on a continuous variables.
NA[5] – Not applicable, because our analysis didn't include the calculation of the risk of exposure.

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