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Changes in airway inflammation and remodeling in swimmers after quitting sport competition.

Short running title: Swimmers' airway changes after quitting sport competition.

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To the Editor,

Intense swimming training in chlorinated pools affects the structure of the airways in elite swimmers.¹ Regardless of the magnitude of airway responsiveness, airway inflammation and remodeling are similar to those of non-athletes with mild asthma. Airway structural changes in swimmers are mainly characterized by increased mast cell and eosinophil numbers, collagen deposition, and sub-epithelial basement membrane thickness.¹ A partial reversibility of airway hyperresponsiveness (AHR) has been reported in competitive swimmers after a rest or retirement period.^{2,3} The present study aimed at comparing inflammation and remodeling in competitive swimmers' airways before and 2 years after retirement from competitive sport.

The study protocol was approved by the Institutional Ethics committee (CER20159) and registered at www.clinicaltrials.gov (NCT00686452). Twenty-one elite swimmers who signed a written informed consent and had bronchial biopsies during their training career were solicited about 2 years after retirement from sport competition and six of them accepted a second bronchoscopy (*for subjects' selection details see Figure E1 in the Appendices*). Bronchial biopsies obtained when they were competitive swimmers (PRE) and 2-years after retirement (POST) (23 ± 3 months) were then analyzed.¹ The subjects had an exhaled nitric oxide (FeNO) measurement, allergy skin prick tests, blood sampling for immunoglobulin E (IgE) measurement, methacholine inhalation test, eucapnic voluntary hyperpnea challenge and, on a separate day, a bronchoscopy with bronchial biopsies. Biopsies from both periods were taken by the same pulmonologist (M.L.) according to the methodology previously described¹ and analysed by experienced thoracic pathologists (*see Material and Methods E1 in the Appendices*).

Characteristics of the six swimmers are presented in Table E1 (*see Appendices*). Lung function at both visits is presented in Table 1. Swimmers A and E had about 12% decrease in baseline forced expiratory volume in one second (FEV₁) from pre- to post-career. Post-career, swimmers A and B had no more AHR to methacholine inhalation test, whereas swimmer F developed AHR (PC₂₀<16mg.mL⁻¹) and hyperventilation-induced bronchoconstriction (HIB) to eucapnic voluntary hyperpnea challenge, and swimmer C HIB. Biopsies scheduling are presented in Table E2 (*see Appendices*). The results of cell counts measured by immunohistochemistry are presented on Figure 1. Lymphocyte (CD3) and neutrophil (elastase) counts significantly decreased from pre- to post-training career in all swimmers (p<0.05). Collagen III deposition increased in all swimmers' post-career except for swimmer A (p=0.039). No significant trend was observed for eosinophil and mast cell number and tenascin staining (Figure 1). Post-career severity of HIB and biopsy mast cell counts correlated significantly (r²=0.81, p=0.01).

To our knowledge, our study is the only report on airway structural changes in elite swimmers, during and after quitting their competitive career. Our results highlight a decrease in lymphocyte and neutrophil numbers two years after retirement in all swimmers showing a reversibility of bronchial mucosa inflammation, most likely due to intense training cessation. There is no such evidence for mast cell and eosinophil counts, for which the variations seem at least partly related to those of airway function and responsiveness. On one hand, in addition to the correlation observed between HIB and mast cell count post-career, the change between both visits of these two parameters are in the same direction in all but swimmer E, being in favour of an implication of mast cells in HIB.⁴ On the other hand, eosinophil counts increased post-career in swimmers A and E, who also showed a decrease in baseline FEV₁ of about 12% after retirement. Both parameters may be linked in all likelihood, as in asthma, during a 5-year follow-up, the persistence of eosinophils in induced sputum has previously been associated to the decrease in FEV₁.⁵ As previously observed,¹ AHR in swimmers does not appear to be eosinophil driven, and despite no direct evidence, maybe

due to different time-course,^{6,7} airway reactive changes may have rather been associated with neutrophilic/lymphocytic infiltrates.

Finally, swimmers C and F had a worsening of their airway responsiveness after retirement. We could not exclude that a transient increased airway inflammatory process occurred between the two visits, as both still swam 4 years. It may have promoted the development of AHR and HIB in swimmer F, and a more permanent HIB in swimmer C resulting from the recovery process, as previously observed in reactive airway dysfunction syndrome.⁶

In all but one swimmer (swimmer A), collagen III was increased post-career, contrasting to what is observed in the case of antigen avoidance or reactive airway dysfunction syndrome' morphological changes after exposure cessation.^{6,7} High-molecular weight hyaluronan (HMW-HA) is a source of collagen III, promoting fibrotic process and attenuating inflammatory response, exercise-induced bronchoconstriction, lung injury and even ozone-induced AHR.⁸ The potential of degradation of HMW-HA into low molecular weight-HA (LMW-HA), favored by oxidative stress, hydric disbalance and biomechanical stretch of fibroblasts, epithelial and endothelial cells may promote inflammatory process, and AHR.⁸ Therefore, induction of HMW-HA following intense training and chlorine exposure cessation, may have a reparative effect on lungs while intense swimming in chlorinated pools during many years of training may degrade HMW-HA into LMW promoting inflammatory process and AHR, as shown in studies on cigarette smoke.⁹ The lack of correlation between collagen and airway function, responsiveness or inflammation, probably reflects the different time-course of recovery process, observed for AHR and inflammation.⁸

In conclusion, despite variability of some parameters between individuals, changes of airway inflammatory process were reversible post-career in swimmers. Changes in inflammatory cell numbers between both periods probably reflect the change in airway function and/or responsiveness that occurred during this period. Care should be taken regarding those results, as only 6 swimmers were followed post-career. However, it provides interesting data on training

cessation airway changes in swimmers and requests further studies aiming at understanding reversible fibrosis processes in swimmers, notably the increase in collagen III post-career and the potential role played by HMW- and LMW-HA.

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Table 1. Swimmers' individual data

Swimmers	Sex	FEV ₁ (% pred.)			FVC (% pred.)			PC ₂₀ (mg/mL)		EVH (% fall in FEV ₁)		FeNO (ppb)		Exercise-induced symptoms (yes:no)		Atopy (yes:no)
		PRE	POST	% change	PRE	POST	% change	PRE	POST	PRE	POST	PRE	POST	PRE	POST	PRE/POST
A	M	104	92	-11.5	111	112	0.9	3.2	>16	16	15	27.8	19.5	Yes	Yes	Yes
B	F	129	138	7.0	135	144	6.7	9.7	>16	8.8	3.9	53.7	41.5	Yes	No	Yes
C	F	109	121	11.0	130	128	-1.5	3.3	1.3	9.1	37.3	19.9	41.6	Yes	Yes	Yes
D	F	135	124	-8.1	138	123	-10.9	>16	>16	6.5	6.3	16.2	18.3	No	No	No
E	M	122	106	-13.1	125	117	-6.4	>16	>16	8.7	7.2	NA	26	No	No	No
F	M	112	113	0.9	123	129	4.9	>16	6.9	7	11.3	7.6	15	No	No	No

Each swimmer is identified by a letter (from A to F). PRE: first biopsy during the swimming career; POST: second biopsy after 2 years of competitive sport retirement; M: male; F: female; NA: not available; FeNO: fractional exhaled nitric oxide; FEV₁: Forced expiratory volume in one second; FVC: forced vital capacity; EVH (% fall in FEV₁): Maximum percentage fall in FEV₁ after the eucapnic voluntary hyperpnea challenge; PC₂₀: Concentration of methacholine inducing a 20% fall in FEV₁; Exercise-induced symptoms are defined as the complaint of breathlessness, wheezing or cough during effort. Atopy was defined as at least one positive reaction to skin prick tests (wheal diameter ≥mm) among 26 common aeroallergens, in the presence of a negative saline control and positive histamine. None had a diagnosis of asthma during childhood or before beginning the competitive swimming career. Swimmers A, B and C took a bronchodilator and antihistamines when needed, and subject B also had a leukotriene receptor antagonist during the PRE-period. Only swimmer A took bronchodilator and antihistamine drugs when needed in the POST-period.

Figure legend

Figure 1: Quantification of inflammatory cells (a, b, d and e), collagen III deposition in the subepithelial membrane (c), thickness of tenascin immunoreactive band (f) and basement membrane thickness (g) in bronchial biopsies of swimmers (n=6).

Each subject is represented by a letter (from A to F).

Appendices :

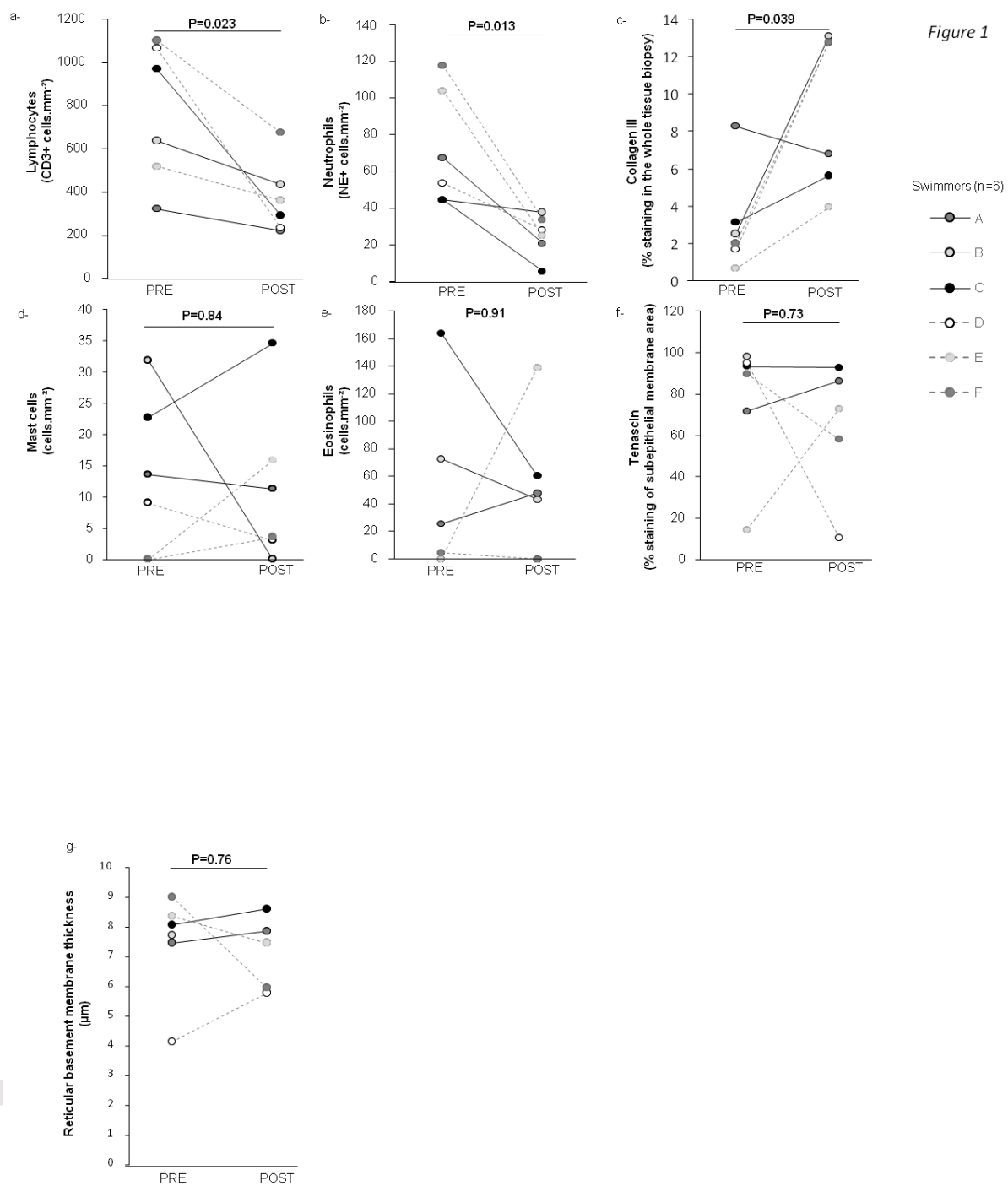
Figure E1: Flow of participants through each stage of the study.

Nine of the swimmers were not in the Quebec City region anymore and did not want to come back for the tests, two were still competitive swimmers, two were excluded (one used daily nasal corticosteroids, one was a regular smoker since the cessation of the competition), and one refused to have a second bronchial biopsy sampling. Among the remaining 7 subjects, one had no more material from the first biopsies for analysis.

Table E1: Anthropometric data and training habits of swimmers

Table E2: Swimmers' individual data for the time between both biopsies and serum IgE values

Each subject is represented by a letter (from A to F).



Supplementary Material

Material and Methods E1:

Immunohistochemistry: From the formaldehyde fixed-paraffin embedded blocks, 4.0- μ m-thick sections were cut on a microtome and placed on charged slides. Mouse monoclonal antihuman CD3 (Dako, Mississauga, Ont. Can.), neutrophil elastase (NE, Dako), type-III collagen (Abcam, Toronto, Ont., Can.), tenascin-C (Novocastra, Leica biosystems, Concord, Ont., Can.), and mucin 5A (Cedarlane, Burlington, Ont., Can.) were used as primary antibodies. All immunohistochemistry (IHC) protocol steps were performed using Dako Autostainer Link38 (Dako Agilent Pathology Solutions, Santa Clara, CA) with reagents from Dako EnVisionTM FLEX+ kit. Counter-staining was carried out using Harris's hematoxylin. For eosinophil and mast cell counts (including mast cells infiltration in airway smooth muscle), Giemsa stain was performed on the biopsy slides after deparaffinization and rehydration, following a standard cytology protocol combining both May-Grunwald and Giemsa stains (Harleco, EMD Chemicals Gibston, N.J). Cell counts were performed by trained pathologists (P.J. and P.O.). One biopsy from each subject was analyzed pre and post retirement for each parameter. Several zones of the biopsies were evaluated, and the mean value was used for analyses.

Slides Digitization and Digital Analysis of Images: IHC-stained slides were digitalized using a slide scanner (NanoZoomer 2.0-HT; Hamamatsu, Bridgewater, NJ, USA) and digital biopsy images with 20x magnification were obtained and visualized with the software NDP.view2 (Hamamatsu, Bridgewater, NJ). They were analyzed by the image analysis software CaloPix (developed and distributed by TRIBVN, Chatillon, France). First, the Random Forest Tree tool was used to select only representative tissue zones creating a Tissue Recognition

algorithm that was applied on whole slide images using the Tissue Recognition for ROA (3.1.0) tool. Tissue selection was visually revised by an independent observer and all the necessary corrections were made based on the Hematoxylin&Eosin corresponding slides to ensure maximum precision. IHC analysis was then performed on the resulting images using Calopix “Immuno Surface 3.0.0” tool. Briefly, an IHC algorithm was developed, and four staining intensity classes (0 = none, +1= weak, +2 = moderate, +3 = strong) were set by an observer with IHC interpretation experience. As a result, the software generated the following data: 1) Total Tissue Area in μm^2 (TTA) of tissue of interest; 2) Positive Segmented Tissue Area in μm^2 (PSTA); 3) Percentage of Positive Segmented Tissue Area (PPSTA) that is corresponding to the ratio between PSTA/TTA; 4) Percentage of each IHC staining category 0, 1+, 2+, 3+. IHC digital results were visually revised by an independent observer. Data were then exported into an Excel file for further interpretation. Cases with less than 30% of viable tissue were not included in the analyses. IHC staining for Tenascin-C and Collagen III antibodies were analyzed as described above. The reticular basement membrane thickness was expressed as the mean of 5 measurements.

For CD3 antibody analyses, after slide digitalization as previously described, slide digital images were imported by Calopix software and Tissue Recognition for surface quantification tool was applied to quantify the tissue surface area (mm^2). IHC analysis was then performed on the slide digital images using Calopix “Immuno Object 2.10.10” tool. Briefly, an IHC algorithm was developed, and four staining intensity classes (0 = none, +1= weak, +2 = moderate, +3 = strong) were set by an observer with IHC interpretation experience. All cells that presented staining above +1 were counted as positive cells for CD3 antibody. As a result, the software generated the number of

positive cells by tissue surface area. IHC digital results were visually revised by an independent observer. Data were then exported into an Excel file for further interpretation. Cases with less than 30% of viable tissue were not included in the analyses.

Figure E1 *Flow of participants through each stage of the study.*

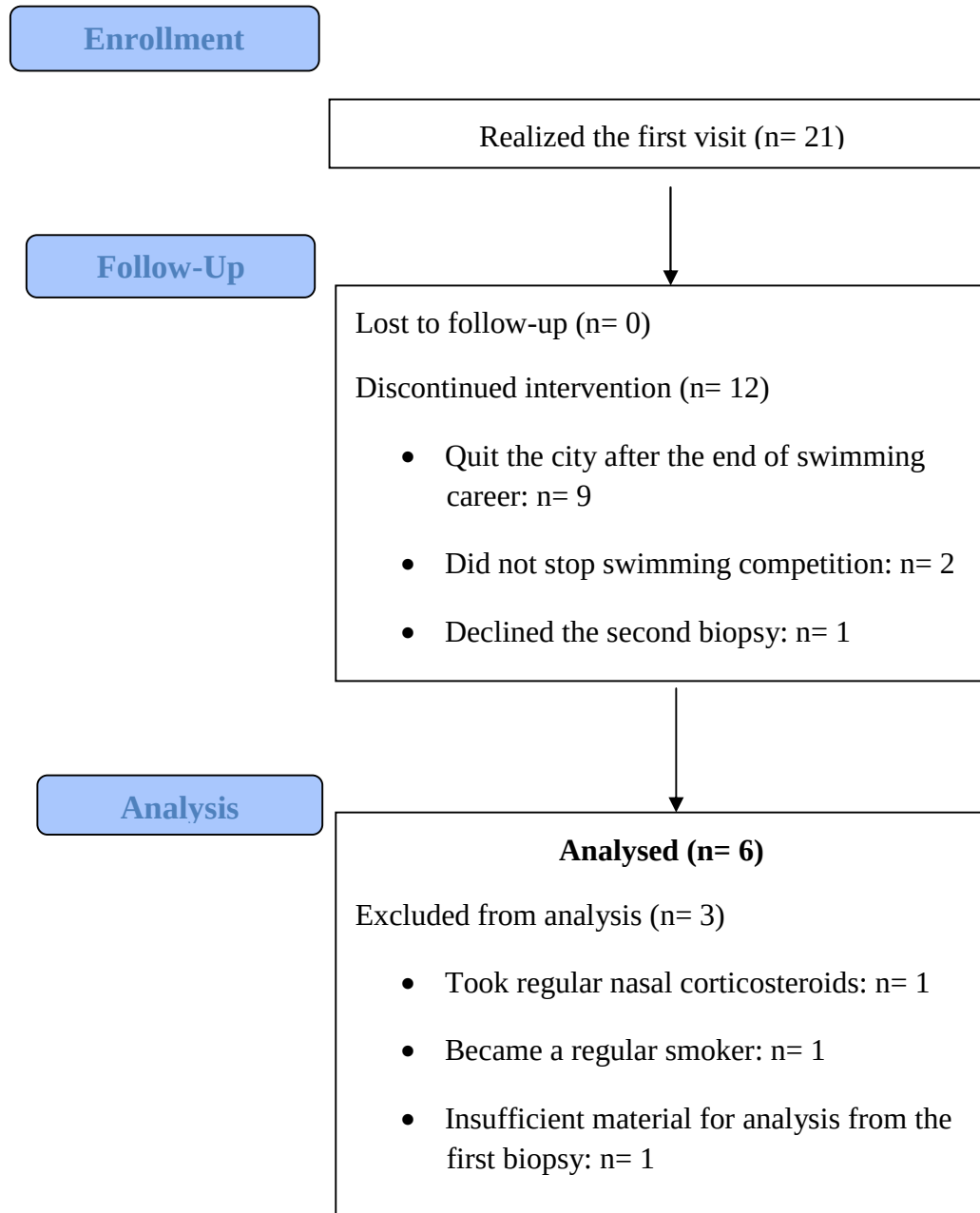


Table E1 *Anthropometric data and training habits*

Characteristics (n=6)	PRE	POST	P
Age (years)	20±1	25±1	<0.001
BMI (kg.m ⁻²)	24±3	25±4	0.28
Swimming (years)	12±3	14±4	-
Swimming (hours.week ⁻¹)	17±4	2±1	<0.001
Other sports training (hours.week ⁻¹)	4±3	4±3	0.98
FEV ₁ (% pred.)	119±12	116±16	0.58
FVC (% pred.)	127±10	126±11	0.70

BMI: body mass index; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity.

Table E2 Swimmers' individual data for biopsies scheduling and serum IgE values

Swimmers	Training before the first visit (years)	Time between both biopsies (years)	Remaining swimming career after the first biopsy (years)	Serum IgE (IU.mL ⁻¹)	
				PRE	POST
A	11	3	1	1000	388
B	12	3	1	46	43
C	17	6	4	39	47
D	13	4	2	33	30
E	8	5	3	31	25
F	8	6	4	NA	57

Each swimmer is identified by a letter (from A to F). PRE: first biopsy during the swimming career; POST: second biopsy after 2 years of competitive sport retirement. NA: not available.