IS CHILDHOOD ASTHMA ASSOCIATED WITH BIOLOGICAL AGING MARKERS?

UNIVERSITAT AUTONOMA DE BARCELONA

OFFICIAL MASTER IN ZOONOSIS AND ONE HEALTH

COURSE OF 2017-2018

Author:

Ana Belén Guzmán Sandoval

Directors:

Lea Maitré, ISGlobal.

Maribel Casas, ISGlobal.

Tutor:

Laila Darwich, Universitat Autónoma de Barcelona.

UNIVERSITAT AUTONOMA DE BARCELONA

Official Master in Zoonosis and One Health

Course of 2017-2018

IS CHILDHOOD ASTHMA ASSOCIATED WITH BIOLOGICAL AGING MARKERS?

Author: Ana Belén Guzmán Sandoval

Directors:

Lea Maitre, ISGlobal. **Maribel Casas, ISGlobal.**

Tutor:

Laila Darwich, Universidad Autónoma de Barcelona.

Acknowledgements

I would first like to thank Lea Maitré and Maribel Casas from IsGlobal who trusted me to handle this research and work and who guided me while allowing me to learn during the time spent in the making of this text.

I would also like to thank IsGlobal and the Helix project for their labor in researching and providing more knowledge regarding diseases and their effect on population, as well as all the families who take part in this studies and who offer such valuable data.

Content

Abstract 1

1. Introduction 2

2. Objective 4

3. Materials and Methods 4

3.1 Study design and population 4

3.2 Sample collection 5

3.3 Measurement of aging markers 5

3.4 Childhood wheezing and asthma 7

3.5 Confounders 7

3.6 Statistical Analysis 7

3.6.1 Population description and confounders selection 7

3.6.2 Association between wheezing and asthma and biological aging markers 8

4. Results 8

4.1 Characteristics of the study population 8

4.2 Bivariate analysis 11

4.3 Association between active wheezing and ever asthma and biological aging markers 15

5. Discussion 16

6. Conclusion 18

7. References 19

Figures and Tables content

Figure 1. Population flow chart. 5
Table 1. Population description. 10
Table 2. Bivariate analysis 12
Table 3. Adjusted regression models for asthma phenotype and biological aging markers. 16

Abstract

Background: Asthma, the most common chronic disease in childhood, is an inflammatory disease of the airways. The presence of chronic inflammation in the organism causes production of ROS; this suggests that asthma could be associated to an accelerated biological aging.

Objective: To explore if there is an association between asthma in school-age children from the population-based birth cohort study HELIX (n = 1623) and three proposed aging markers: telomere length, mitochondrial DNA, and DNA methylation age.

Methods: Information on wheezing and asthma was obtained from parental questionnaires administered at 6-11 years. We defined "active wheezing" as either by wheezing or by having taken medication in the last year and "asthma" as children ever diagnosed with asthma by a doctor. Leukocyte TL and mtDNA content were measured in the children at 6-11 years employing real time polymerase chain reaction (qPCR). DNAm was measured on blood samples using the Horvath (Illumina HumanMethylation27 Bead Chip) and Hannum (Illumina HumanMethylation 450 BeadChip) algorithms.

Results: Prevalence of active wheezing and asthma was 10% and 17%, respectively. Ever asthma was borderline statistically significantly associated with a decrease of 2.21% (95% CI: -6.21%;1.94%) in mtDNA. mtDNA also decreased in children with active wheezing compared to children with no wheezing. Active wheezing was non-statistically significantly associated with an increase in HOma (0,20 (95% CI: -0,03;0,44) and HAma (0,28 (95% CI: -0.13;0.68), in relation to children without active wheezing. Ever asthma was non-statistically significantly associated with an increase in HAma compared with children without asthma. Associations did not substantially change after considering air pollution as confounder.

Conclusion: This study, the first one to assess whether asthma is related to biological aging in children, finds little evidence of associations of active wheezing and ever asthma with three different biological ageing markers. Further studies are needed to confirm these results.

1. Introduction

Asthma is an increasingly more common chronic inflammatory disease of the airways in both children and adults worldwide. ISAAC (The International Consensus Report on the Diagnosis and Treatment of Asthma) showed that the global prevalence increased from mid-1990s to mid-2000s, also being seen to increase as population becomes urbanized (I. Asher & Pearce, 2014; Masoli, Fabian, Holt, & Beasley, 2004). The symptoms are usually related to airway obstruction and increased airway responsiveness to various stimuli provoking: "wheezing", shortness of breath, chest tightness and coughing (I. Asher & Pearce, 2014). Asthma is worsened by a combination of genetic and environmental factors such as air pollution and tobacco smoke (I. Asher & Pearce, 2014; Masoli et al., 2004; Sears et al., 2003). Individuals who suffer from asthma at early ages are more likely to suffer asthma in adulthood, whether it is by relapse or persistence (Masoli et al., 2004; Sears et al., 2003). In addition, childhood asthma is of particular concern since it can predispose to other health problems (i.e. repeated respiratory infections) and chronic illnesses as COPD throughout life.

Asthma involves a variety of inflammatory cell types and pro-inflammatory mediators which can cause oxidative stress with the production of reactive oxygen species (ROS) (Liu et al., 2009). ROS arising from an asthma chronic inflammation state cause molecular damage in signaling pathways, enzymatic functions, gene expression and other essential biological processes. Depending on the individuals coping mechanisms to this damage, patients show different reaction to treatment, severity and persistence of the disease (Jesenak, Zelieskova, & Babusikova, 2017). Asthma is a heterogeneous condition and many different phenotypes have been defined (Wenzel, 2006). Indeed, asthma phenotype needs to be better understood based on the oxidative stress and inflammatory pathways. There is evidence (Jesenak et al., 2017) that individuals with persistent asthma may experience more profound molecular damage, but this is little understood, although crucial for the clinics to better understand the aetiology of asthma and subsequent comorbidities and persistence. Furthermore, the relation between a known chronic inflammatory disease that has a high prevalence in the population and its effect on the aging mechanisms has received little attention.

Different markers have been found to measure accelerated ageing at the molecular level. Telomeres are DNA structures that, with the help of telomere specific proteins (telomerase),

protect the end of chromosomes and maintain integrity of the genome (Albrecht et al., 2014). Cell senescence involves shortening of telomeres since with every cell division the telomere is not fully replicated (Epel et al., 2004; Kyoh et al., 2013). This erosion can also be caused by environmental factors, especially exposure to ROS arising from chronic inflammatory diseases or tobacco smoke exposure. Telomere length (TL) determinants can also include heritability, sex (women have on average longer telomeres), ethnicity and socioeconomic status (Zhu, Belcher, & van der Harst, 2011). TL has been widely studied as a marker for biological age in association with many health conditions, although not to our knowledge in the context of asthma and TL in children.

Another biological aging marker proposed is mitochondrial DNA content. It is well known that mitochondria's main function is to produce energy (ATP) for the cell through the respiratory chain (Wei, 2000). This organelle also fulfills functions of signaling transduction for cell proliferation, apoptosis, calcium storage and metabolism (Clemente et al., 2016). Mitochondria counts with its own set of DNA (mtDNA) that consists of 13 polypeptides related to activities of respiration and oxidative phosphorylation (Wei, 2000). Mitochondria are both the main source of ROS (normal by-product of oxidative phosphorylation) and the primary target of oxidative stress (especially mtDNA since it lacks protection that is present in nuclear DNA) (Clemente et al., 2016; Wei, 2000). Because of the conditions mentioned before, it is also used as a marker of biological age.

More recently, DNA methylation (DNAm) of some genomic regions that can be hyper or hypo methylated during aging has been found to be a good biomarker for cell and tissue aging (Horvath, 2013). It is a score made from a combination of CpG sites related to genes involved in age related diseases such as Alzheimer's disease, cancer, tissue degradation, DNA damage, and oxidative stress (Hannum et al., 2013). The difference between DNAm age and chronological age has been suggested as a marker of age acceleration and it can be caused by environmental (i.e. stress), disease (i.e. cancer) or inherited factors (Gao, Zhang, Breitling, & Brenner, 2016; Hannum et al., 2013; Horvath, 2013). A study done in children has shown that there are hypo methylated sites directly related to tobacco exposure and disease in children (Gao et al., 2016). Another wide association (EWAS) study in children searched for the relation between asthma and DNAm (Arathimos et al., 2017). This has opened the path for more studies that relate the mechanisms of disease associated with aging in children and perhaps suggest effects carried through adulthood.

Current research has focused on asthma and biological aging markers separately. Only recently, investigation on relation between biological age and asthma has been done in adults (Belsky et al., 2014) and the before mentioned EWAS study for children and asthma (Arathimos et al., 2017) offering a need to determine whether children are being affected by this association.

2. Objective

The present study aims to examine whether childhood asthma is associated with three different aging markers (TL, mtDNA, and DNAm) in a large birth-cohort study population with children between the ages of 6 and 11 years.

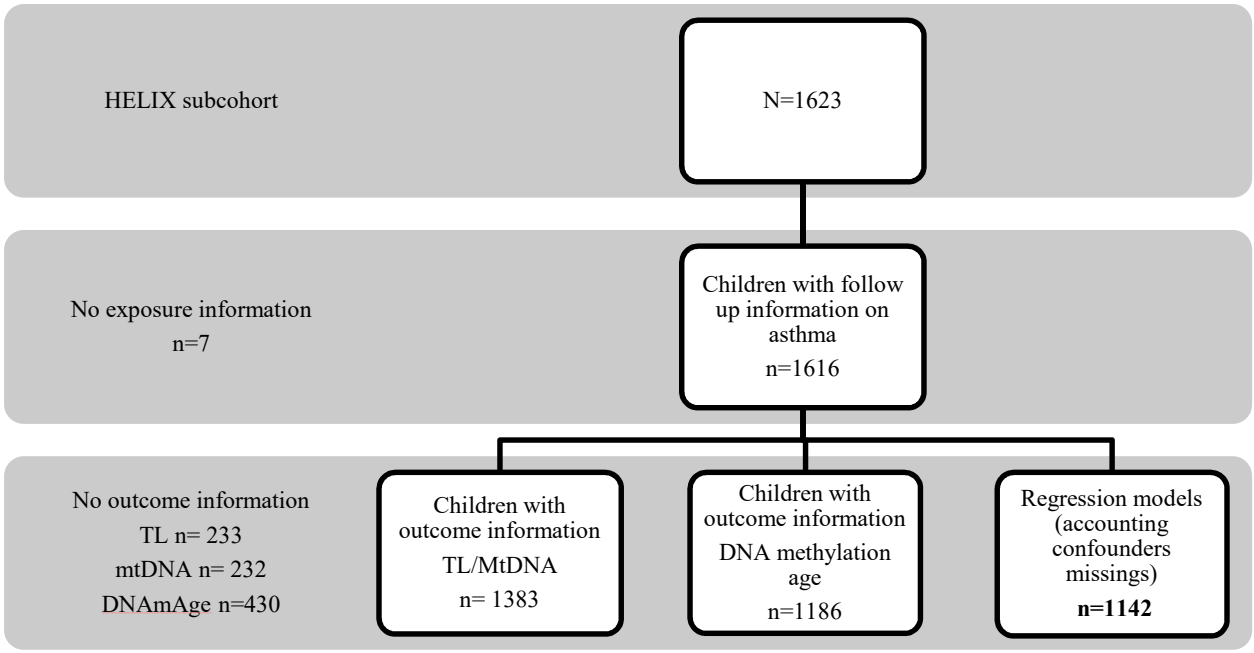
3. Materials and Methods

3.1 Study design and population

The HELIX (Human Early Life Exposome) study is a collaborative project of six population-based birth cohorts in different European countries: United Kingdom (BiB: Born in Bradford), France (EDEN; Étude des Déterminants pré et postnatals du développement et de la santé de l'Enfant), Spain (INMA; Infancia y Medio Ambiente), Lithuania (KANC; Kaunus cohort), Norway (MoBa; Norwegian Mother and Child Cohort Study), and Greece (RHEA; Mother Child Cohort study in Crete).

The six cohorts have in total a population comprising 31,472 mother child pairs, with data on environmental exposures, clinical outcomes, social factors, diet, etc. From this population, approximately 200 mother-child pairs were selected randomly from each cohort to produce a nested subcohort (HELIX) of *n*=1301 mother-child pairs with a new follow up at 6-11 years where clinical examination and biological samples were taken. Some cohorts then invited and examined 322 further subjects making the subcohort population N=1623 (Vrijheid et al., 2014, Maitré, et al. in press). The study population was restricted to children with information on wheezing or asthma and TL, mtDNA, and DNAm. We defined two populations: one with information on wheezing/asthma and TL/mtDNA (*n*=1383) and a second one with wheezing/asthma and DNAm data (n=1186). Some missings were identified for confounders which are detailed in Figure 1.

Figure 1. Population flow chart.



3.2 Sample collection

The follow-up examination of the subcohort consisted of collecting new biological samples according to the planned omics analyses (6-11 years children). Eighteen mL of blood were collected at the end of the clinical examination of each child, ensuring approximately 3 hour fasting. Blood samples were collected using a butterfly vacuum clip and local anesthetic and processed into a variety of sample matrices for serum, plasma, whole blood for RNA extraction (Tempus tubes – Life Technologies Cat No: 4342792), red cells, and buffy coat for DNA extraction. After processing, these samples were frozen at -80°C under optimized and standardized procedures (Maitré, et al. in press; Vrijheid et al., 2014).

3.3 Measurement of aging markers

TL and mtDNA were attained in the Center for Environmental Sciences, Hasselt University, Diepenbeek, Belgium; DNAm was obtained in the Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Barcelona, Spain

Average relative TL was measured by a modified quantitative real-time PCR (qPCR) protocol as described previously (Cawthon, 2009). Telomere and single copy-gene reaction mixture and PCR cycles used were described elsewhere (Martens et al. 2016). All measurements were performed in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 384-well format. In each run, a 6-point serial dilution of pooled DNA was run to assess PCR efficiency that ranged from 90% to 102%; as well as eight inter-run calibrators to account for the inter-run variability. Relative TLs were calculated using qBase software (Biogazelle, Zwijnaarde, Belgium). They were expressed as the ratio of telomere copy number to single-copy gene number (T/S). We achieved coefficients of variation (CV) within triplicates of the telomere runs, single-copy gene runs, and T/S ratios of 0.84%, 0.43%, and 6.4%, respectively.

Relative mtDNA content was measured by determining the ratio of two mitochondrial gene copy numbers (mitochondrial encoded NADH dehydrogenase subunit 1 (MT-ND1) and mitochondrial forward primer for nucleotide 3212 and reverse primer from nucleotide 3319 (MTF3212/R3319)) to one single-copy nuclear control genes (acidic ribosomal phosphoprotein P0 (RPLP0)) using real-time PCR. mtDNA and single copy-gene reaction mixture and PCR cycles used can be found elsewhere (Janssen et al., 2012, Clemente et al., 2016). All measurements were performed in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 384-well format. On each run, a 6-point serial dilution of pooled DNA was run to assess PCR efficiency that ranged from 96% to 107% as well as eight inter-run calibrators to account for the inter-run variability. Relative mtDNA content (two mitochondrial gene copy numbers relative to single nuclear gene) was calculated using qBase software (Biogazelle, Zwijnaarde, Belgium). CV within duplicates was 0.72% for mitochondrial genes and 0.43% for the single-copy gene. These measures were summarized by only two variables in total.

Methylation age acceleration variables were estimated using the Horvath (Horvath, 2013) and Hannum (Hannum et al., 2013) algorithms from DNA methylation data (blood DNA methylation quantified using 450K, Illumina).
The Horvath method was developed using a dataset of roughly 4000 samples from 20 healthy tissues and cell types assembled using a combination of Illumina HumanMethylation27 BeadChip (27K) and Illumina Human Methylation 450 Bead Chip (450K)

technology. The Horvath method uses 353 CpG sites which are common to both platforms. The Horvath method uses 353 CpG sites and the Hannum method uses 71 CpG sites to estimate age. More details can be found in https://labs.genetics.ucla.edu/horvath/dnamage/. Variables for methylation age had 2 measures: AAHOAdjCellCounts (HOma) and AAHAAdjCellCounts (HAma). These were measures of age acceleration adjusted for cell counts (residuals resulting from multivariate regression models that regress an estimate of DNA methylation age on chronological age and various blood immune cell counts). AAHOAdjCellCounts and AAHAAdjCellCounts correspond to age acceleration measures based on Horvath (2013) and Hannum (2013), respectively.

3.4 Childhood wheezing and asthma

Information on occurrence of wheezing and asthma was obtained through questionnaires adapted from the International Study on Asthma and Allergy in Childhood (ISAAC) filled by the parents at the time of follow up (6-11 years children) (M. I. Asher et al., 1995). Active wheezing wad defined as having had any episode of wheezing or had medication for asthma in the last year. Asthma was defined as asthma ever diagnosed by a doctor.

3.5 Confounders

Confounders considered for the statistical analyses were: child's sex, child's age at the time of the follow-up, socioeconomic status (maternal education, family affluence score (FAS), parity, child ethnicity, cohort center, child's body mass index score (BMI in kg/m²-for-age-and-sex z-scores using the international World Health Organization (De Onis et al., 2007) reference curves in order to allow comparison with other studies, zBMI, birth weight in grams, tobacco smoke exposure during pregnancy (TSEp), current tobacco smoke exposure from parents smoking habit at age of follow up (TSEc), marital status, mother age at birth, parents asthma history, batch effect (only for TL), cell proportion (only for TL and mtDNA since methylation age markers were already adjusted for cell count) and concentrations of air pollutants (NO_2, PM_{10}, $PM_{2.5}$).

3.6 Statistical analysis

3.6.1 Population description and confounders selection

First, description of the populations was done according to the whole population N=1383 and according to wheezing and asthma statuses. Bivariate analysis tests (Wilcoxon, Chi square, Student's t-test, Kruskal-Wallis or correlation depending on variable characteristics) were carried

out to explore the associations between wheezing/asthma and biological aging markers with potential confounders. Confounders were selected based on a review of the literature on the determinants of childhood wheezing and asthma and biological aging makers (Belsky et al., 2014; Clark et al., 2010; Strachan & Cook, 1998). Secondly, we performed linear regression models between biological aging markers and chronological age of the children (6-11 years) adjusted for cohort. Third, we also tested the association across the three aging markers to see how they associate to each other using linear regression models adjusted to sex, cohort and cell count.

3.6.2 Association between wheezing and asthma and biological aging markers

The distribution of TL/mtDNA (both continuous) was checked and log10 transformed to reach normality. HOma and HAma variables were both normally distributed. Active wheezing and asthma were analyzed as predictors of accelerated aging response through the four biological markers separately. For each association (i.e. active wheezing and TL, active wheezing and mtDNA), four linear regression models were constructed considering the confounders associated with wheezing or asthma and at least one aging marker in the bivariate analyses. The four models were the following: 1) minimally adjusted model (sex, age, cohort), 2) moderately adjusted model (minimally adjusted model + zBMI, ethnicity, FAS, TSEp), 3) fully adjusted model (moderately adjusted model + cell count – only for TL and mtDNA), and 4) fully adjusted model + air pollution (fully adjusted model + NO_2). Beta coefficient units for TL correspond to the ratio explained previously, in which a β under 1 corresponds to a shorter telomere and over 1 to a longer telomere. Similarly, for mtDNA, β under 1 corresponds to less quantity of mtDNA and over 1 more. Since TL and mtDNA were log10 transformed they were back transformed for interpretation as percentage using the formula ((10^(Beta))-1)*100 and ((10^(CI))-1)*100. All analyses were performed using the statistical package RStudio Version 1.1.423 – © 2009-2018 RStudio, Inc. http://www.r-project.org).

4. Results

4.1 Characteristics of the study population

Table 1 describes the characteristics of the study population of all children. For this description we focused on the large population, since distribution was similar for the smaller population subset. Children had a median age of 8.2 years (6.5-9.1), 635 (46%) were female, mainly of white European origin 1208 (87%). Median family affluence scale (FAS) showed 687

(50%) were from high income families with 624 (48%) of mothers with high education, 609 (47%) were first time mothers, 217 (17%) actively smoked during pregnancy and 427 (31%) were passive smokers during pregnancy; 545 (59%) of parents were active smokers at the age of follow up. Birth weight median was 3360g (3040-3670) and 1079 children (78%) were considered as normal weight at follow up evaluation.

Table 1. Population description.

	Missing %	All		Missing %	All		Missing %	All
N		1383			1383			1383
Child characteristics			**Family characteristics**			**Other**		
Sex, female %	0%	46%	**Family Affluence Score, %**	4 (0.0%)		**Cell proportion, median (IQR)**	209 (15.0%)	
Age (years), median (IQR)	0%	8,2 (6,5-9,1)	Low		11%	NK		0,016 (0-0,048)
Body mass index, %	0%		Middle		39%	Bcell		0,109 (0,082-0,138)
Obese		6%	High		50%	CD4T		0,19 (0,15-0,232)
Overweight		16%	**Maternal education, %**	90 (6.5%)		CD8T		0,116 (0,086-0,144)
Underweight/Normal		78%	Low		15%	Eos		0 (0-1,03e-18)
zBMI, median (IQR)		0,38 (-0,35-1,22)	Middle		33%	Mono		0,076 (0,059-0,093)
Birth weight (g), median (IQR)	59 (4%)	3360(3040 - 3670)	High		45%	Neu		0,463 (0,381-0,543)
Ethnicity, %	2 (0.001%)		**Parity, %**	79 (6.0%)		**Air contaminants, median (IQR)**	4 (0.0%)	
Other		13%	No child		44%	NO_2		807 (386 - 1214)
White european		87%	One child		34%	PM_{10}		828 (434 - 1210)
Doctor diagnosed asthma, yes %	0%	10%	More than one child		16%	$PM_{2.5}$		817 (396 - 1196)
Active wheezing in the last year %	0%	17%	**Marital status %**	62 (4.0%)				
TL, median (IQR)	0%	0,99(0,88-1,13)	Living alone		3%			
mtDNA, median (IQR)	0%	1,03(0,89-1,21)	Living with father		90%			
HOma, median (IQR)	0%	-0,11(-0,97-0,85)	Other		2%			
HAma, median (IQR)	0%	-0,08(-1,73-1,79)	**Mother age at birth, median (IQR)**	62 (4.0%)	31(27.5 - 34)			
			Tobacco smoke exposure during pregnancy %	94 (6.0%)				
			No exposure		47%			
			Passive smoker		31%			
			Active smoker		16%			
			Parents smokers age 9 years, %	16 (1.0%)				
			Yes		59%			
			Parents asthma %	3 (0.0%)				
			Yes		19%			

4.2 Bivariate analysis

Table 2 shows p-values of bivariate analyses between wheezing/asthma and biological aging markers with potential confounders. Shadowed in grey are those associations with significant p-values. Confounders that showed association with wheezing or asthma and at least one aging marker were: child's sex, child's age, zBMI, ethnicity, FAS, TSEp, blood cell count (eosinophils and Bcells), and NO_2. mtDNA showed the most associations to confounders.

Table 2. Bivariate analysis

	Ever asthma	No asthma	Asthma (p-value)	Wheezing	No wheezing	Wheezing (p-value)	TL (p-value)	mtDNA (p-value)	HOma (p-value)	HAma (p-value)
N	144	1239		241	1142		1383	1383	1186	1186
Child characteristics										
Sex, female %	35%	47%	0,006	37%	48%	0,002	0,000	0,575	0,045	0,008
Age (years), median (IQR)	7,2(6,5-8,8)	8,2(6,5-9,1)	0,198	6,9(6,5-8,8)	8,3(6,6-9,2)	0,000	0,141	0,000	0,927	0,631
Body mass index, %			0,723			0,029	0,298	0,176	0,434	0,234
Obese	6%	6%		10%	5%					
Overweight	18%	15%		16%	16%					
Underweight/Normal	76%	78%		74%	79%					
zBMI, median (IQR)	0,39(-0,27-1,4)	0,38(-0,35-1,21)	0,462	0,53(-0,27-1,44)	0,35(-0,36-1,2)	0,052	0,003	0,460	0,097	0,478
Birth weight (g), median (IQR)	3420(3052 - 3750)	3360(3040 - 3660)	0,288	3305(2992 - 3670)	3370(3050 - 3670)	0,232	0,626	0,000	0,961	0,122
Ethnicity, %			0,043			0,025	0,380	0,000	0,999	0,240
Other	18%	12%		17%	12%					
White European	81%	88%		83%	88%					
Doctor diagnosed asthma, yes %	100%	0%	-	43%	4%	0,000	0,578	0,047	0,569	0,424
Active wheezing in the last year %	72%	11%	0,000	100%	0%	-	0,952	0,001	0,104	0,304
TL	0,98(0,88 - 1,11)	0,99(0,88-1,13)	0,578	0,98(0,89-1,13)	0,99(0,88-1,12)	0,952	-	0,005	-	-
mtDNA	0,99(0,86-1,19)	1,04(0,89-1,22)	0,047	1(0,857 - 1,16)	1,04(0,9-1,23)	0,001	0,005	-	-	-
HOma, median (IQR)	-0,02(-0,88 - 0,94)	-0,12(-0,99 - 0,84)	0,000	0,13(-0,85-0,94)	-0,14(-1,03-0,80)	0,000	-	-	-	0,000
HAma, median (IQR)	0,22(-1,54-2,15)	-0,08(-1,74-1,77)	0,000	0,16(-1,65-1,95)	-0,11(-1,75-1,76)	0,000	-	-	0,000	-

Family characteristics										
Family Affluence Score, %			0,983			0,001	0,182	0,000	0,357	0,729
Low	11%	11%		10%	11%					
Middle	40%	39%		49%	37%					
High	49%	50%		40%	52%					
Maternal education, %			0,761			0,304	0,552	0,000	0,045	0,351
Low	17%	15%		16%	15%					
Middle	32%	33%		37%	32%					
High	44%	45%		41%	46%					
Parity, %			0,805			0,190	0,049	0,144	0,755	0,937
No child	44%	44%		42%	44%					
One child	33%	34%		32%	35%					
More than one child	18%	16%		20%	15%					
Marital status, %	97,92%	95%	0,155			0,110	0,017	0,000	0,283	0,256
Living alone	1,39%	3%		2%	3%					
Living with father	92,36%	90%		90%	90%					
Other	4,17%	2%		4%	2%					
Mother age at birth, median, IQR	30,5(26,5-33,5)	31(27,7-34,1)	0,061	30,7(27-34,2)	31(27,8-34)	0,297	0,001	0,097	0,871	0,411
Tobacco smoke exposure during pregnancy, %			0,043			0,019	0,224	0,003	0,009	0,025
No exposure	56%	46%		41%	48%					
Passive smoker	23%	32%		39%	29%					
Active smoker	16%	16%		14%	16%					
Parents smokers age 9 years, %			0,526			0,091	0,040	0,016	0,216	0,312
Yes	37%	40%		44%	38%					
Parents asthma, %			0,000			0,000	0,458	0,208	0,885	0,875
Yes	44%	16%		30%	17%					

	Ever asthma	No asthma	Asthma (p-value)	Wheezing	No wheezing	Wheezing (p-value)	TL (p-value)	mtDNA (p-value)	HOma (p-value)	HAma (p-value)
Other										
Cell proportion, median, IQR										
NK	0,009(0-0,047)	0,017(0-0,049)	0,146	0,012(0-0,045)	0,017(0-0,049)	0,173	0,034	0,000	-	-
Bcell	0,113(0,085-0,141)	0,108(0,081-0,137)	0,061	0,114(0,085-0,142)	0,107(0,0811-0,137)	0,033	0,000	0,000	-	-
CD4T	0,198(0,16-0,238)	0,189(0,148-0,23)	0,078	0,187(0,157 - 0,233)	0,19(0,149-0,231)	0,630	0,000	0,000	-	-
CD8T	0,11(0,078-0,145)	0,116(0,087-0,144)	0,401	0,117(0,090-0,146)	0,115(0,086-0,144)	0,603	0,000	0,000	-	-
Eos	6,12e-20(0-2,58e-18)	0(0-8,25e-19)	0,000	0(0-2,5e-18)	0(0-7,41e-19)	0,000	0,187	0,063	-	-
Mono	0,073(0,057-0,088)	0,076(0,059-0,093)	0,085	0,075(0,056-0,089)	0,076(0,060-0,093)	0,170	0,286	0,931	-	-
Neu	0,456(0,375-0,541)	0,463(0,383-0,543)	0,369	0,459(0,376-0,55)	0,463(0,383-0,543)	0,524	0,000	0,000	-	-
Air contaminants, median, IQR										
NO_2	734(302 - 998)	819(394 - 1236)	0,010	861(374 - 1224)	788(388 - 1214)	0,531	0,569	0,001	0,480	0,218
PM_{10}	648(371 - 1270)	841(454 - 1204)	0,258	900(483 - 1245)	814(420 - 1198)	0,083	0,916	0,036	0,468	0,424
$PM_{2.5}$	890(556 - 1190)	803(386 - 1198)	0,168	774(376 - 1089)	824(398 - 1219)	0,126	0,816	0,000	0,161	0,925

Tests carried out according to variable characteristics: Chi square, Wilcoxon, Kruskal, Correlation, T student test.

4.3 Association between active wheezing and ever asthma and biological aging markers

Table 3 shows the results of the linear regression models associating wheezing and asthma with each aging biomarker adjusting for confounders selected from the bivariate analysis.

Ever asthma was borderline statistically significantly associated with a decrease of 2,21% (95% CI: -6,21%;1,94%) in mtDNA content, compared to children without asthma. This association became stronger but still non-significant when adjusting by cell count (-3,03% (95% CI: -6,47%;0,54%)) and did not substantially change after including NO_2 into the model (-2,89% (95% CI: -6,34%;0,69%)). mtDNA also decreased in children with active wheezing compared to children with no wheezing (Fully adjusted model: -1,36% reduction; 95% CI: -4,30%;1,66%).

Active wheezing was non-statistically significantly associated with an increase in HOma (0,20 (95% CI: -0,03;0,44) and HAma (0,28 (95% CI: -0.13;0.68), in relation to children without active wheezing. These associations remained stable after considering air pollution. Ever asthma was non-statistically significantly associated with an increase in HAma (0,18 (95% CI: -0,30;0,67)), compared with children without asthma.

No associations were observed between active wheezing/ever asthma and any of the biological aging markers.

Table 3. Adjusted regression models for asthma phenotype and biological aging markers.

N=1142	Minimally adjusted*			Moderately adjusted**			Fully adjusted***			Fully adjusted + NO_2****		
Biological aging marker	β	95% CI		β	95% CI		β	95% CI		β	95% CI	
TL												
Active wheezing	1,16%	-1,59%	3,99%	1,35%	-1,40%	4,17%	1,51%	-1,25%	4,34%	1,51%	-1,25%	4,34%
Diagnosed asthma	0,14%	-3,11%	3,51%	0,44%	-2,81%	3,80%	0,24%	-3,01%	3,60%	0,28%	-2,97%	3,65%
mtDNA												
Active wheezing	-1,81%	-5,17%	1,66%	-1,48%	-4,87%	2,02%	-1,36%	-4,30%	1,66%	-1,37%	-4,30%	1,66%
Diagnosed asthma	-2,21%	-6,20%	1,94%	-1,92%	-5,93%	2,26%	-3,03%	-6,47%	0,54%	-2,89%	-6,34%	0,69%
HOma							**Not applicable**					
Active wheezing	0,17	-0,06	0,40	0,20	-0,03	0,44				0,20	-0,03	0,44
Diagnosed asthma	0,10	-0,17	0,37	0,09	-0,19	0,37				0,09	-0,19	0,37
HAma							**Not applicable**					
Active wheezing	0,24	-0,16	0,64	0,28	-0,13	0,68				0,27	-0,13	0,68
Diagnosed asthma	0,21	-0,27	0,69	0,18	-0,30	0,67				0,20	-0,28	0,69

*Adjusted for age, sex, and cohort, **Adjusted for age, sex, cohort,,zBMI, ethnicity, FAS, and TSEp, ***Adjusted for age, sex, cohort,zBMI, ethnicity, FAS, TSEp, and cell count (only for TL and mtDNA), ****Adjusted for age, sex, cohort,,zBMI, ethnicity, FAS, TSEp, cell count (only for TL and mtDNA), and NO_2.

5. Discussion

In this study, based on six European countries, we observed a borderline inverse association between ever asthma and mtDNA. We also observed that active wheezing was associated with an increase in HOma and HAma, although associations did not reach statistical significance. Neither active wheezing nor ever asthma were associated with TL.

To our knowledge, only one study has previously assessed the relationship between asthma and biological aging markers and it was conducted in adults (Belsky et al., 2014). In this study, a two time point analysis of TL in a population of n=1037 individuals that were sought for asthma at 9 assessments spanning 9-38 years was carried out. They observed that adults with life-course-persistent asthma had shorter TL ($\beta = 20.31$; $P < 0.001$) than those with no reported asthma, and this association was attenuated when adjustment for eosinophils was performed (Belsky et al., 2014). No changes in TL were observed in adults with childhood and adolescent/adult onset asthma. In this study TL was the only aging marker considered. Our study, in contrast, includes TL and two more biological aging markers. This allowed a better understanding of the role asthma

plays on the organism at the molecular level in particular in inflammatory cells. The effect caused by higher eosinophils seen by Belsky, et al. could not be assessed in our study since no association between asthma and TL was shown. Nonetheless, in our study eosinophils were also seen to be slightly higher in asthma diagnosed children. Some of the children in our study might have adolescent onset of asthma, showing importance of following studies in the population to add to the understanding of persistence effect of the disease on molecular levels. Perhaps, children with asthma in our study might need more time to show noticeable molecular changes on aging markers. It is therefore important to do follow up studies in children that participated in HELIX during adolescence and adulthood so that changes in biological aging markers can be identified. It was also seen by another study that age at onset was related to persistence in adulthood which contributes to the importance of including age of diagnosis and beginning of symptoms in the analysis (Sears et al., 2003). This will contribute to a better understanding of the effect of persistent asthma at molecular levels and also the asthma phenotype which can also follow different inflammatory cell association and not just an eosinophilic pattern, but also neutrophilic or pauci-granulocytic (Wenzel, 2006).

DNA methylation studies have shown association between asthma/wheezing and DNA methylation sites in children, but when the authors adjusted for cell count all associations were lost, showing also a high influence of eosinophils and neutrophils on the results (Arathimos et al., 2017). It was suggested that asthma phenotype may be better defined in subtypes according to their eosinophils and neutrophils status (Wenzel, 2006).

The main strengths of this study are the large sample size and the availability of three different biological aging markers. We also could adjust our models for cell counts and air pollution, which is one of the most potential confounders of the association of asthma-related symptoms and biological aging markers. Our study however, has some limitations: a lack in ethnic diversity, as 87% of children were from white European origin, un-inclusion of dietary elements that could influence oxidative stress by intake of antioxidants that could help as coping mechanisms and a simple definition of asthma that did not include other important factors as repeated infectious respiratory diseases and time of diagnosis or onset of symptoms that would indicate persistence. Adding to this, first, wheezing and asthma were self-reported and therefore outcome misclassification cannot be ruled out due to under- or over-reporting of wheezing and

asthma. This may have led to either attenuation or overestimation of the effect estimates. Second, it is important to consider that mtDNA showed a high sensitivity to external factors and that causes difficulty to analyze the effect caused by asthma. Finally, since data on wheezing and asthma was collected at the same time as aging markers determination, reverse causation cannot be ruled out (i.e. if shorter TL predisposes the organism to suffer from asthma or if asthma is shortening TL).

6. Conclusion

Childhood asthma is an increasingly prevalent disease that affects the quality of life, predisposes to other diseases and could become persistent through adulthood. This study, the first one to assess whether asthma is related to biological aging in children, finds little evidence of associations of active wheezing and ever asthma with three different biological ageing markers. The overall lack of molecular damage obtained in this study indicate that there is a chance for treatment and prevention for asthmatic children at school-age to avoid or diminish damages due to asthma related oxidative stress. However, future studies should look at different subtypes of asthma (severity, persistence, and age of onset) to confirm our results.

7. References

Albrecht, E., Sillanpää, E., Karrasch, S., Alves, A. C., Codd, V., Hovatta, I., … Schulz, H. (2014). Telomere length in circulating leukocytes is associated with lung function and disease. *The European Respiratory Journal*, *43*(4), 983–92. https://doi.org/10.1183/09031936.00046213

Arathimos, R., Suderman, M., Sharp, G. C., Burrows, K., Granell, R., Tilling, K., … Relton, C. L. (2017). Epigenome-wide association study of asthma and wheeze in childhood and adolescence. *Clinical Epigenetics*, *9*(1), 1–16. https://doi.org/10.1186/s13148-017-0414-7

Asher, I., & Pearce, N. (2014). Global burden of asthma among children. *The International Journal of Tuberculosis and Lung Disease : The Official Journal of the International Union against Tuberculosis and Lung Disease*, *18*(11), 1269–78. https://doi.org/10.5588/ijtld.14.0170

Asher, M. I., Keil, U., Anderson, H. R., Beasley, R., Crane, J., Martinez, F., … Williams, H. C. (1995). International study of asthma and allergies in childhood (ISAAC): rationale and methods. *European Respiratory Journal*, *8*(3), 483–491. https://doi.org/10.1183/09031936.95.08030483

Belsky, D. W., Shalev, I., Sears, M. R., Hancox, R. J., Harrington, H. L., Houts, R., … Caspi, A. (2014). Is chronic asthma associated with shorter leukocyte telomere length at midlife? *American Journal of Respiratory and Critical Care Medicine*, *190*(4), 384–391. https://doi.org/10.1164/rccm.201402-0370OC

Cawthon, R. M. (2009). Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Research*, *37*(3), 1–7. https://doi.org/10.1093/nar/gkn1027

Clark, N. A., Demers, P. A., Karr, C. J., Koehoorn, M., Lencar, C., Tamburic, L., & Brauer, M. (2010). Effect of early life exposure to air pollution on development of childhood asthma. *Environmental Health Perspectives*, *118*(2), 284–290. https://doi.org/10.1289/ehp.0900916

Clemente, D. B. P., Casas, M., Vilahur, N., Begiristain, H., Bustamante, M., Carsin, A. E., … Nawrot, T. S. (2016). Prenatal ambient air pollution, placental mitochondrial DNA content,

and birth weight in the INMA (Spain) and ENVIRONAGE (Belgium) birth Cohorts. *Environmental Health Perspectives*, *124*(5), 659–665. https://doi.org/10.1289/ehp.1408981

De Onis, M., Onyango, A. W., Borghi, E., Siyam, A., Nishida, C., & Siekmann, J. (2007). Development of a WHO growth reference for school-aged children and adolescents. *Bulletin of the World Health Organisation*, *85*(10), 812–819. https://doi.org/10.2471/BLT.

Epel, E. S., Blackburn, E. H., Lin, J., Dhabhar, F. S., Adler, N. E., Morrow, J. D., & Cawthon, R. M. (2004). Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences*, *101*(49), 17312–17315. https://doi.org/10.1073/pnas.0407162101

Gao, X., Zhang, Y., Breitling, L. P., & Brenner, H. (2016). Relationship of tobacco smoking and smoking-related DNA methylation with epigenetic age acceleration, *7*(30).

Hannum, G., Guinney, J., Zhao, L., Zhang, L., Hughes, G., Sadda, S., … Friend, S. (2013). Resource Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. *Molecular Cell*, *49*(2), 359–367. https://doi.org/10.1016/j.molcel.2012.10.016

Horvath, S. (2013). DNA methylation age of human tissues and cell types. *Genome Biol*, *14*(10), R115. https://doi.org/10.1186/gb-2013-14-10-r115

Janssen, B. G., Munters, E., Pieters, N., Smeets, K., Cox, B., Cuypers, A., … Nawrot, T. S. (2012). Placental Mitochondrial DNA Content and Particulate Air Pollution during in Utero Life. *Environmental Health Perspectives*, *120*(9), 1346–1352. https://doi.org/10.1289/ehp.1104458

Jesenak, M., Zelieskova, M., & Babusikova, E. (2017). Oxidative Stress and Bronchial Asthma in Children—Causes or Consequences? *Frontiers in Pediatrics*, *5*(July). https://doi.org/10.3389/fped.2017.00162

Kyoh, S., Venkatesan, N., Poon, A. H., Nishioka, M., Lin, T. Y., Baglole, C. J., … Hamid, Q. (2013). Are leukocytes in asthmatic patients aging faster? A study of telomere length and disease severity. *Journal of Allergy and Clinical Immunology*, *132*(2), 64–68. https://doi.org/10.1016/j.jaci.2013.02.010

Liu, L., Poon, R., Chen, L., Frescura, A.-M., Montuschi, P., Ciabattoni, G., … Dales, R. (2009).

Acute Effects of Air Pollution on Pulmonary Function, Airway Inflammation, and Oxidative Stress in Asthmatic Children. *Environmental Health Perspectives*, *117*(4), 668–674. https://doi.org/10.1289/ehp11813

Maitré, L. (in press). Cohort Profile: the Human Early Life Exposome (HELIX) study - A European Population-Based Exposome Cohort. *BMJ Open in Cohort profiles.*

Martens, D. S., Plusquin, M., Gyselaers, W., De Vivo, I., & Nawrot, T. S. (2016). Maternal pre-pregnancy body mass index and newborn telomere length. *BMC Medicine*, *14*(1), 1–10. https://doi.org/10.1186/s12916-016-0689-0

Masoli, M., Fabian, D., Holt, S., & Beasley, R. (2004). The global burden of asthma: Executive summary of the GINA Dissemination Committee Report. *Allergy: European Journal of Allergy and Clinical Immunology*, *59*(5), 469–478. https://doi.org/10.1111/j.1398-9995.2004.00526.x

Sears, M. R., Greene, J. M., Willan, A. R., Wiecek, E. M., Taylor, D. R., Flannery, E. M., … Poulton, R. (2003). A Longitudinal, Population-Based, Cohort Study of Childhood Asthma Followed to Adulthood. *New England Journal of Medicine*, *349*(15), 1414–1422. https://doi.org/10.1056/NEJMoa022363

Strachan, D. P., & Cook, D. G. (1998). Parental smoking and childhood asthma : longitudinal and case-control studies Parental smoking and childhood asthma : longitudinal and case-control studies, (July 2005), 204–212.

Vrijheid, M., Slama, R., Robinson, O., Chatzi, L., Coen, M., van den Hazel, P., … Nieuwenhuijsen, M. J. (2014). The human early-life exposome (HELIX): Project rationale and design. *Environmental Health Perspectives*. https://doi.org/10.1289/ehp.1307204

Wei, H. L. Y. (2000). Mitochondrial Role in Life and Death of the Cell, *112*, 2–15.

Wenzel, S. E. (2006). Asthma : defining of the persistent adult phenotypes. *Lancet*, *368*, 804–13.

Zhu, H., Belcher, M., & van der Harst, P. (2011). Healthy aging and disease: role for telomere biology? *Clinical Science*, *120*(10), 427–440. https://doi.org/10.1042/CS20100385