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Tetanus vaccination is associated with differential DNA-methylation: Reduces the risk of asthma in adolescence

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ABSTRACT

Background: Vaccinations have been suggested to be associated with increased risk of allergic diseases. Tetanus vaccination is one of the most frequently administered vaccines as a part of wound management and was also found to be associated with increased serum IgE levels. We hypothesized that the vaccination modifies the risk of allergic diseases through epigenetic changes such as DNA methylation.

Method: Data on tetanus vaccination between 10 and 18 years of age was collected from a birth cohort established on the Isle of Wight UK in 1989. DNA methylation data were collected from individuals at different ages (at birth [n = 30], age 10 [n = 34], age 18 [n = 245] and during pregnancy [n = 121]) using the Illumina Infinium HumanMethylation450 K array. Firstly, we performed an epigenome-wide screening to identify cytosine-phosphate-guanine sites (CpGs) associated with tetanus vaccination in 18-year-olds. Secondly, we tested their association with asthma, allergic sensitization, eczema, serum IgE and pulmonary lung function (FVC, FEV1, FEV1/FVC, and FEF25-75%). We then described changes in the methylation of the selected CpG sites over age, and by vaccination status.

Results: Tetanus vaccination was found to be associated with decreased methylation of cg14472551 (p value 0.5×10^{-5}, FDR-adjusted p value 2.1×10^{-4}) and increased methylation of cg01669161 (p value 0.0007, FDR-adjusted p value 0.014). Both CpGs, in turn, were associated with decreased risk of asthma at 18 years of age. Cg14472551 is located in an intron of KIAA1549L, whose protein binds to a B-cell commitment transcription factor; cg01669161 is located between an antisense regulator of the proteasome assembly chaperone PSMG3, and TFAMP1, a pseudogene. Increased methylation of cg01669161 was also associated with decreased serum IgE levels.

Conclusion: DNA methylation changes following tetanus vaccination may offer a novel prospect to explain a differential occurrence of asthma in adolescence.

1. Introduction

Vaccination is related to widespread immunity and eradication of many infectious diseases [1,2]. Vaccines induce acquired active immunity, which initiates a cascade of differentiation of immune cells into effector and memory cells [3,4]. Vaccines, along with booster doses generate immune memory cells to maintain long-lasting or lifelong protection against a specific antigen [4,5]. The tetanus vaccine has adjuvant effects and has been associated with upregulating total and specific immunoglobulin E (IgE) antibodies [6,7], which in turn play a vital role in allergic diseases.

Effects of vaccination on the prevalence of allergic diseases remain in debate. To assess their association, several studies have been conducted [7–12]. Scheduled childhood vaccination of DTP (diphtheria, tetanus, pertussis) has been reported to be associated

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with increased risk of asthma [10]. Similar findings were reported in children and adolescents (2 months to 16 years of age), concluding that DTP and tetanus vaccination (TVac) were associated with increased risk of allergies [7]. Conversely, the DPPT (Diphtheria, Polio, Pertussis, Tetanus) and MMR (measles, mumps, and rubella) vaccines were not linked with physician diagnosed asthma or eczema in children in one birth cohort study [11]. A lack of associations was also reported in a population-based cohort study (TAHS-Tasmanian Longitudinal Health Study), which followed participants from 7 to 45 years of age [9]. Thus, whether allergy and asthma are influenced by vaccination remains in contention.

Epigenetic processes such as DNA methylation (DNA-M) may provide a mechanistic link between vaccination and the occurrence of disease. DNA-M, the addition of a methyl group to a cytosine followed by a guanine (CpG sites, CpGs), is known to mediate the effect of environment at transcriptional levels, and also has well-evidenced connections to allergic disease [13]. An immunological study suggested that epigenetic changes in vaccine-induced memory CD4 and/or CD8 T cells act as on-off-on switches for immune responses after re-exposure to antigen [14]. A recent study demonstrated an overall decrease in global DNA-M in vaccinated compared to non-vaccinated chickens [15].

Asthma is a common chronic inflammatory disease of the airways characterized by wheezing, chest tightness and shortness of breath. It has been suggested that DNA–M may modulate the regulation of genes that are associated in the pathogenesis of asthma and allergy [13,16–19].

We hypothesized that TVac could alter the methylation of specific CpGs, influencing allergic disease risk. We performed an epigenome-wide association study (EWAS) to identify CpGs differentially methylated between 18-year old children vaccinated and non-vaccinated between 10 and 18 years. Next, we analyzed whether tetanus vaccination-associated CpGs are associated with allergy and asthma. To further explore the potential effect of TVac, we describe differential DNA-M of specific CpGs at different ages (at birth, age 10, 18 years and during pregnancy) and across children who were vaccinated and non-vaccinated between 10 and 18 years.

2. Methods

2.1. Study population

A whole population birth cohort was established on the Isle of Wight, UK in 1989 to study the natural history and etiology of asthma and allergic diseases. The study was approved by the local research ethics committee (NRES Committee South Central–Hampshire B). Of 1536 eligible children born between January 1, 1989 and February 28, 1990, 1456 were enrolled after excluding adoptions, infant deaths and refusals. Written informed consents were received from enrolled parents (F_0). Their children (F_1) were followed up with detailed questionnaires at ages 1, 2, 4, 10 and 18 years. Guthrie cards (blood collected within 7 days of birth, n = 30) and peripheral blood samples at age 10 (n = 34) and 18 (n = 370) years were collected from randomly selected subjects for DNA-M profiling. Pregnant F_1 participants and their partners were recruited and followed up at 12, 20 and 28 weeks of pregnancy with detailed questionnaires. During pregnancy, peripheral blood samples from pregnant F1 participants (n = 121) were collected for epigenome-wide DNA-M profiling. Nearly all the children (94.3%) in our cohort have been vaccinated at 10 years of age according to the recommended UK immunization schedule [32].

2.2. DNA methylation

DNA was extracted from Guthrie cards using a procedure based on that described by Beyan et al. [20], and from whole blood using a standard salting out procedure [21]. In all samples up to 1 μg of DNA was bisulfite-treated for cytosine to thymine conversion using the EZ 96-DNA methylation kit (Zymo Research, CA, USA), following the manufacturer's standard protocol. DNA-M was assessed using the Illumina Infinium HumanMethylation450 Beadchip (Illumina, Inc., CA, USA). Arrays were processed using a standard protocol [22], with multiple identical control samples assigned to each bisulfite conversion batch to assess assay variability. Samples were randomly distributed on microarrays to control for batch effects. The beadchips were scanned using a BeadStation, and the methylation level (β value) was calculated for each queried CpG locus using the Methylation module of BeadStudio software. Beta values represent the proportions of methylated (M) over the sum of methylated (M) and unmethylated (U) sites ($\beta = M/[c + M + U]$) with c being a constant to prevent dividing by zero [23].

2.3. Pre-processing DNA-M data

The detection p-values reported by Genome Studio were used as a QC measure of probe performance in our study. CpGs with a detection p-value > 0.01 in > 10% of samples were removed [24]. Bioconductor IMA (Illumina methylation analyzer) and Com-Bat statistical computing packages were used for pre-processing DNA-M data and inter-array variation adjustments respectively [25,26]. We excluded all CpGs with a potential single nucleotide polymorphism (SNP) in the binding region or at base-pair extension according to dbSNP137, because probe-SNPs may interfere with DNA-M measurement (Fig. 1).

2.4. Exposure

Information on vaccination at 18 years was collected from question "Have you had the following immunizations since you were 10 years old?", which included tetanus, BCG, diphtheria, polio and other vaccinations, answered by the individuals at the 18-year follow up.

2.5. Outcome variables

In the first step, we performed epigenome-wide screening for differentially methylated CpGs that were associated with TVac. In the second step, tetanus vaccination-associated CpGs were tested for association with asthma, eczema, and allergic sensitization (Fig. 1). Asthma at age 18 years was defined by "ever had asthma" as well as either "wheezing or whistling in the chest during the previous 12 months" or "current treatment for asthma". Allergic sensitization at age 18 years was determined by positive skin prick test response ($\geqslant$3 mm larger than negative control) using 14 common food and aeroallergens (ALK-Albello, Horsholm, Denmark) [27]. Eczema was defined as chronic or chronically relapsing, itchy dermatitis lasting more than six weeks with characteristic morphology and distribution [28], following Hanifin and Rajka criteria [29]. In the third step, to determine the consistency, we tested disease-related markers such as serum IgE level and lung function measurements at age 18. Serum IgE was assessed using Immunocap (Phadia, Uppsala, Sweden), designed to measure IgE between 2.0 and 1000 kU/L in serum. Regarding lung function, forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), and forced expiratory flow ($FEF_{25\%-75\%}$) were measured using a Koko spirometer and software with a portable desktop device (both PDS Instrumentation, Louisville, KY, USA). Spirometry was performed and evaluated according to the American Thoracic

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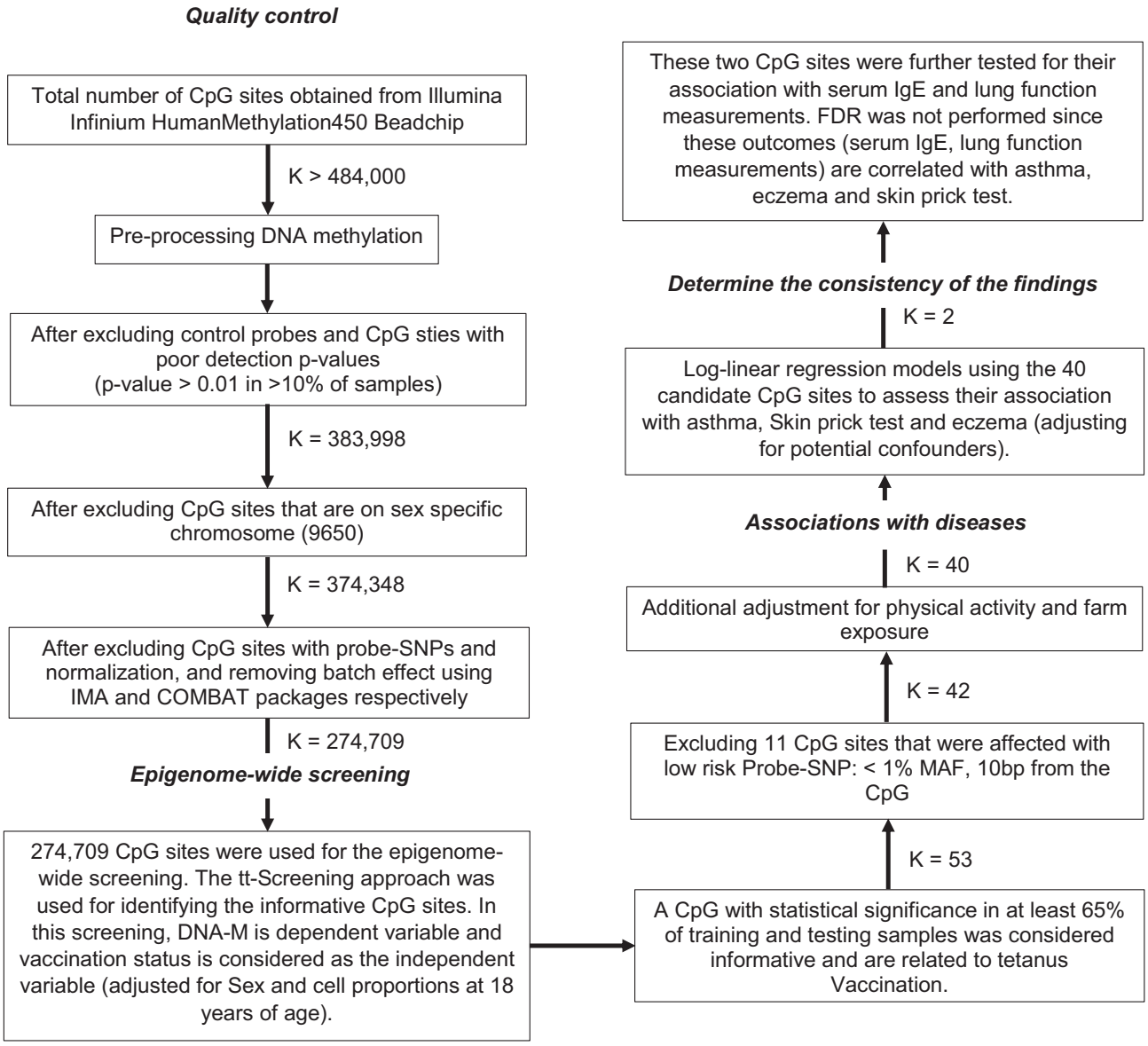

Fig. 1. Flow chart illustrating the identification of CpG sites that are associated with Tetanus vaccination, asthma, eczema, and allergic markers.

Society (ATS) criteria. Children were required to be free of respiratory infection for two weeks, withhold steroids intake and were advised to abstain from any β-agonist medication for six hours and caffeine intake for at least 4 h. The fraction of exhaled nitric oxide (FeNO) was measured (Niox mino, Aerocrine AB, Solna, Sweden) according to ATS guidelines [30] as described by Scott et al. [31].

2.6. Potential confounders

Variables potentially associated with allergic disorders and/or DNA-M were considered to be potential confounders, including gestational smoking, chest infections at age one and two years, duration of breast feeding (in weeks), sex of the child, current smoking status, farm exposure and number of days of exercise at 18 years of age. Regarding lung function, we controlled for sex of the child, gestational smoking, duration of breast feeding (weeks), and height (in centimeters) at 18 years of age. Duration of breast feeding information was collected from the questionnaire answered by F_0 mothers at age 1 and 2 years. Chest infection at age one and two years of ages were defined by "recurrent chest infection one year" and "recurrent chest infections at two years" of age. Information regarding gestational smoking, current smoking status, daily physical activities like number of days of exercise, height of the individuals were obtained from the detailed questionnaire answered at age 18 years follow up. Farm exposure of the individual was ascertained by "Have you ever lived on farm".

2.7. Statistical analysis

The sample analyzed in this work was assessed for its representability of the total cohort. M-values (logit transformed β values) were used for epigenome-wide screening [33]. The ttScreening, R computing package (R version 3.1.1) [34] was applied to screen CpGs that are potentially associated with the status of TVac at 18 years of age. The analytical methods implemented in this computing package facilitates a screening process that filters the non-informative CpGs through 100 iterations of a training-and-testing process with robust regressions. By building a more generalized model than conventional EWAS methods,

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ttScreening is able to detect more true positives than conventional methods [34]. In robust regressions, DNA-M is taken as the dependent variable and TVac and covariates (in this case, the potential confounding variables) are the independent variables. A CpG was selected as an informative site if it showed statistical significance in at least 65% of training and testing samples. CpGs identified as informative were further tested in models including additional confounders to intensively test their association with the status of TVac at 18 years of age. We applied log-linear regression models (GENMOD procedure in SAS 9.4) to estimate risk ratios for asthma, allergic sensitization, and eczema at 18 years of age. To adjust for different sample size contributed by girls and boys we used weight statement and controlled for sex. In all analyses, including EWAS and associations of candidate CpGs with asthma, and other allergic diseases, all p-values were adjusted for false discovery rate (FDR) [35] with a p-value of $\leqslant 0.05$ being statistically significant.

To further illustrate our findings, the CpGs linked with asthma, allergic sensitization and/or eczema were categorized into four groups based on equidistant intervals of methylations across the range of the methylation. To evaluate the functional role of these associations, we investigated whether these CpGs are also linked with lung function measurements and serum IgE levels (log-transformed) at age 18 years controlling for duration of breast feeding, sex, height, physical activity, and smoking status at 18 years of age. To this end, we conducted linear regression models with DNA-M as the dependent variable. All regression analyses (log-linear and linear models) were performed using DNA-M data at 18 years of age (n = 370). EWAS analyses were performed in R (R package 3.1.1 version) log-linear and linear regression were performed using the SAS statistical package, Version 9.4 (SAS Institute, Cary, NC, USA).

Finally, we examined differential methylation of tetanus vaccination-associated CpGs at different ages in the F_1 generation and in vaccinated and non-vaccinated individuals. In this descriptive part covering four periods of life, we had to use different sample sizes of female participants that were available to us: n = 34 at birth, n = 30 at age 10, n = 245 at age 18, n = 121 during pregnancy. In addition, we describe the methylation changes in five women, who participated in all of the above four periods.

3. Results

We found no significant difference between the population analyzed, both female (n = 245) and male participants (n = 125), with methylation data available at 18 years of age with the participants of the whole cohort (750 females and 786 males) (Table 1). Among female and male participants in the subsample, 73.5% and 71.2% had TVac, 19.2% and 22.4% had a history of maternal smoking, 7.0% and 6.4% had chest infection at age one year, and 14.2% and 12.8% of them had asthma at 18 years of age respectively.

A total of 53 CpGs were identified in the EWAS, 11 of which were affected by a probe-SNP and thus were excluded (Fig. 1). After adjusting for physical activity and farm exposure, which may be related to TVac, and controlling for FDR, two further CpGs were excluded and a set of 40 CpGs were retained (Table 2). Of these 40 candidate CpGs, cg14472551 (p value 0.5×10^{-5}, FDR p value

Table 1
Asthma, eczema, allergic sensitization, serum IgE, spirometric measurements, tetanus vaccination and potential confounders at age one, two and 18 years of age.

Factor		Study population			Study population		
		Girls (%) (n = 750)	Population analyzed (%) (n = 245)	p-value	Boys (%) (n = 786)	Population analyzed (%) (n = 125)	p-value
Tetanus vaccination at age 18	Yes	62.1 (466)	73.5 (180)	0.5	63.3 (498)	71.2 (89)	0.14
	No	15.4 (116)	15.9 (65)		10.1 (79)	16.8 (21)	
	Missing	22.4 (168)	10.6 (26)		26.5 (209)	12 (15)	
Maternal smoking status	Yes	25.1 (188)	19.2 (47)	0.054	24.9 (196)	22.4 (28)	0.53
	No	74 (555)	80.4 (197)		74 (582)	76.8 (96)	
	Missing	0.9 (86)	0.4 (1)		1 (8)	0.8 (1)	
Do you currently smoke (at age 18)	Yes	25.6 (192)	25.7 (63)	0.25	22.3 (176)	25.6 (32)	0.79
	No	60.6 (455)	73.8 (181)		57.8 (455)	72 (90)	
	Missing	13.73 (103)	0.41 (1)		19.7 (155)	2.4 (3)	
Chest infection at one years of age	Yes	5.1 (38)	7 (17)	0.32	8.2 (63)	6.4 (8)	0.46
	No	70.1 (526)	85.3 (209)		80 (628)	84.8 (106)	
	Missing	20 (150)	8 (19)		12.1 (95)	8.8 (11)	
Chest infection at two years of age	Yes	9.8 (74)	11.4 (28)	0.63	10.1 (80)	11.2 (14)	0.9
	No	70.1 (526)	72.6 (178)		67.4 (530)	74.4 (93)	
	Missing	20 (150)	15.9 (39)		22.3 (176)	14.4 (18)	
Have you ever lived on farm	Yes	7.1 (53)	7.3 (18)	0.66	5.3 (42)	8 (10)	0.53
	No	78.8 (591)	92.6 (227)		75.5 (594)	90.4 (113)	
	Missing	12.5 (94)	0.5 (2)		19.1 (150)	1.6 (2)	
Asthma	Yes	17.1 (128)	14.29 (35)	0.074	13.1 (103)	12.8 (16)	0.37
	No	70.8 (531)	85.7 (210)		69 (543)	87.2 (109)	
	Missing	12.1 (91)			17.8 (140)		
Allergic sensitization	Yes	21.2 (159)	31 (76)	0.26	24.6 (194)	40 (50)	0.17
	No	38.2 (287)	67.7 (166)		27.1 (213)	58.4 (73)	
	Missing	40.5 (304)	1.2 (3)		48.2 (379)	1.6 (2)	
Eczema	Yes	14.3 (107)	15.1 (37)	0.67	6.8 (54)	92.8 (116)	0.68
	No	73.2 (549)	84.4 (207)		76 (597)	7.2 (9)	
	Missing	12.5 (94)	0.41 (1)		17.1 (135)		
		Mean (5%–95% CI)		p-value	Mean (5%–95% CI)		p-value
Days of exercise (days/week)		2.1 (0–7)	2.5 (0–7)	0.07	3.3 (0–7)	3.8 (0–7)	0.06
Duration of breast feeding (week)		14 (0–40)	15.2 (0–40)	0.16	–	–	
Height (cm)		165 (154.5–175)	165 (154–176)	0.44	178.1 (167–189)	177.4 (167–187.3)	0.29
Serum IgE (kU/L)		283 (−102 to 1351)	226.2 (8.9–1165)	0.56	372.7 (−102 to 032.1)	499.2 (14.8–21233)	0.08
FVC at 18 years of age (L)		3.9 (3.1–4.8)	4 (3.1–5)	0.07	5.3 (4.2–6.6)	5.3 (4.1–6.6)	0.99
FEV1 at age 18 years (L)		3.5 (2.7–4.1)	3.5 (2.8–4.3)	0.08	4.6 (3.6–5.6)	4.6 (3.5–5.6)	0.58
FEV1/FVC at age 18 years		0.87 (0.7–0.9)	0.87 (0.75–0.98)	0.65	0.86 (0.86–0.74)	0.87 (0.77–0.99)	0.59
FEF 25–75% (Liter per second)		3.9 (2.5–5.4)	4 (2.5–5.4)	0.33	4.9 (3.2–6.8)	5.1 (3.4–6.9)	0.42

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Table 2
Estimates of the tetanus vaccination effect of originally selected CpG sites based on ttScreening and additionally controlled of confounders in linear models.

CpG sites	Selection probability	Estimate[#]	p-value	Estimate after adjusting[*]	p-value after adjusting[*]	FDR adjusted p-value
cg11987154	80	0.1788	3.92E−06	0.1994	2.00E−07	1.02E−05
cg16109089	81	−0.1303	6.60E−06	−0.1324	1.00E−05	0.0001
cg00756062	76	0.1927	1.00E−05	0.1921	1.00E−05	0.0001
cg23901896	72	0.2136	1.22E−05	0.2022	3.00E−05	0.0001
cg11709763	72	−0.2054	1.26E−05	−0.2102	3.00E−06	4.79E−05
cg14472551	79	−0.3246	1.26E−05	−0.2974	0.004	0.0053
cg19702771	82	0.1309	1.46E−05	0.1452	1.00E−04	0.0004
cg12149319	70	−0.1466	1.70E−05	−0.1726	3.00E−06	4.79E−05
cg03628403	75	0.1364	2.41E−05	0.0946	0.003	0.0037
cg01669161	67	0.1467	2.84E−05	0.1716	6.00E−04	0.001
cg11902863	67	0.1209	2.96E−05	0.0843	0.034	0.0359
cg10365178	75	0.1528	3.00E−05	0.1021	0.003	0.0041
cg17022393	68	0.1774	3.18E−05	0.1695	7.00E−05	0.0002
cg02874226	73	−0.1696	3.19E−05	−0.1641	4.00E−05	0.0002
cg27393285	70	−0.2855	3.30E−05	−0.2281	5.00E−04	0.001
cg07906078	78	0.2102	3.42E−05	0.1995	6.00E−05	0.0002
cg25965290	76	0.1863	3.44E−05	0.1704	2.00E−04	0.0004
cg24383418	71	0.1239	4.24E−05	0.0869	0.14	0.1401
cg02464866	72	0.1971	4.80E−05	0.1578	0.001	0.0015
cg14225485	65	−0.1525	4.98E−05	−0.1231	0.009	0.0098
cg22455614	71	−0.1192	5.38E−05	−0.0894	0.004	0.0053
cg24926780	75	0.1385	5.50E−05	0.1251	4.00E−04	0.0009
cg21722418	66	0.3205	6.90E−05	0.2934	2.00E−04	0.0004
cg09629053	69	−0.2411	7.07E−05	−0.2416	4.00E−05	0.0001
cg00147244	67	0.1739	7.10E−05	0.1427	0.002	0.0025
cg00777375	67	0.1101	7.47E−05	0.0851	0.006	0.0063
cg05445006	69	0.0963	7.71E−05	0.0571	0.065	0.0663
cg03587557	72	−0.1957	7.83E−05	−0.1695	5.00E−04	0.0011
cg27297043	65	−0.1441	8.27E−05	−0.1304	0.002	0.0024
cg02535684	66	0.2837	8.39E−05	0.2404	6.00E−04	0.001
cg11027772	66	−0.1348	8.61E−05	−0.1202	1.00E−03	0.0015
cg24171875	67	0.101	9.10E−05	0.0585	0.028	0.0303
cg25119654	73	−0.1635	0.0001	−0.1824	4.00E−05	0.0001
cg01844176	69	−0.2019	0.00011	−0.1991	2.00E−04	0.0005
cg11196333	67	0.2486	0.00012	0.2525	1.00E−04	0.0004
cg11304283	69	−0.1999	0.00015	−0.1987	6.00E−04	0.001
cg00639041	70	−0.1107	0.00016	−0.1014	5.00E−04	0.001
cg20409368	65	−0.2266	0.00017	−0.2014	0.001	0.0015
cg26525486	65	0.1941	0.00019	0.1453	0.005	0.0062
cg02926601	66	0.1875	0.00022	0.2021	1.00E−04	0.0004
cg16027403	66	−0.1324	0.00025	−0.1195	7.00E−04	0.0011
cg10893220	65	0.1291	0.00029	0.1188	5.00E−04	0.0011

[#] Statistically controlled for sex and cell proportions (CD4, granulocytes, eosinophils, B-lymphocytes, natural killer cells, monocytes) at 18 years of age.
[*] Statistically controlled for sex, farm exposure and physical activity at 18 years of age.

2.1×10^{-4}), cg01669161 (p value 0.0007, FDR p value 0.014) were both statistically significant and associated with asthma status at 18 years. TVac was associated with the increased methylation of cg01669161 and decreased methylation of cg1447251. Both CpGs are in turn associated with decreased risk of asthma.

To demonstrate the relative risk for asthma related to differential methylation of these two CpGs, we categorized each CpG in four groups using equidistant intervals of methylations across the range of the methylation (Table 3). Methylation of cg14472551 (methylation (β value) > 0.55) increased the relative risk of asthma RR = 4.74 (95% CI 1.91–11.75, p = 0.0008) (Table 3). For cg01669161, a decreased methylation (methylation (β value) < 0.45) was associated with increased relative risk of asthma RR = 2.7 (95%CI 1.2–6.41 p = 0.016, Table 3). In addition, decreased methylation of cg01669161 was associated with increased serum IgE (log10) levels (Estimate (SE): −2.48 (1.23), p = 0.045).

We further inspected methylation changes of cg14472551 and cg01669161 at different ages in five female participants with complete follow-up data on DNA-M. In particular, we compared the methylation of these two CpGs among vaccinated (n = 269) and non-vaccinated individuals (n = 60) using the median methylation values (β values). In this descriptive analyses (Fig. 2) we observed that methylation of cg14472551 was higher at birth (β value > 0.6) than at 10 (β value < 0.44) and 18 years (β value < 0.43). Cg14472551 showed the lowest methylation during pregnancy (β value < 0.34). Cg14472551 was lower methylated in vaccinated individuals (n = 269, β value < 0.43) while higher methylated in non-vaccinated individuals (n = 60, β value < 0.47). cg01669161 also showed a difference among vaccinated and non-vaccinated individuals: higher methylation was observed in vaccinated (β value > 0.49) compared to non-vaccinated individuals (β value > 0.47). However, cg01669161 was lower methylated at birth (β value < 0.36), but higher at age 18 (β value > 0.42) and during pregnancy (β value > 0.46).

To further illustrate the effect of the TVac associated CpGs on the prevalence of asthma, we dichotomized the methylation at the median level. Interestingly, the prevalence of asthma among vaccinated individuals (13%) was lower compared to non-vaccinated (22%) however not statistically significant (p = 0.07) (Fig. 3). When we stratified this association by cg14472551 (low vs. high methylated), we found a higher prevalence of asthma among higher methylated children with no vaccination (p = 0.041). In vaccinated children, no increased prevalence of asthma was detected. For cg01669161 the prevalence of asthma was lower among children with higher methylation; however, using a crude dichotomization, the difference did not gain statisti-

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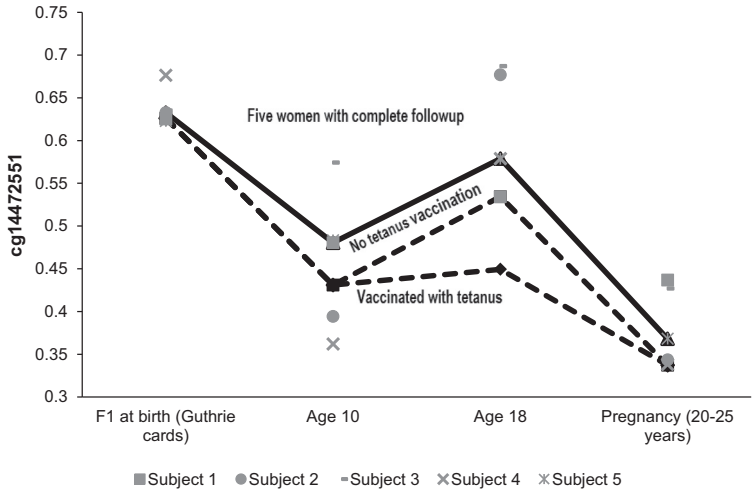
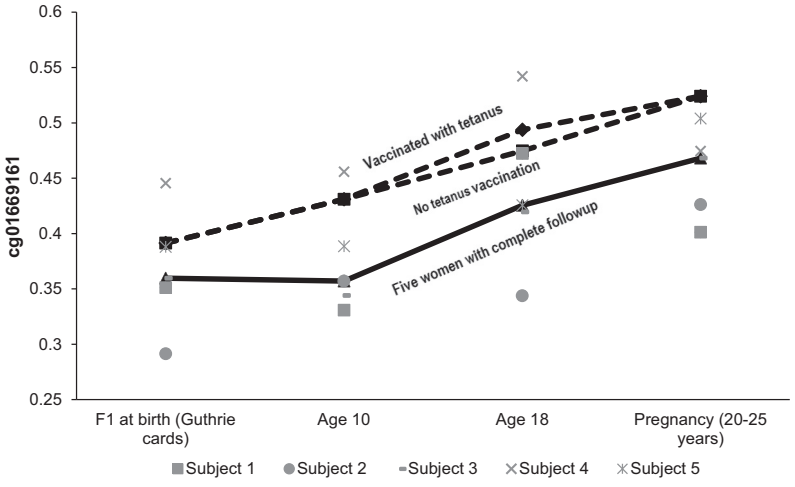
Table 3
Effect of methylation of cg14472551 and cg01669161 on asthma, serum IgE and lung function measurements at 18 years of age.

cg14472551				cg01669161			
Proportion of methylation	Risk ratio	95% CL[+] Asthma[*]	p-value	Proportion of methylation	Risk ratio	95% CL[+] Asthma[*]	p-value
0–0.35 (n = 70)	Reference			0–0.45 (n = 88)	2.78	1.2–6.41	0.016
0.35–0.45 (n = 139)	0.91	0.32–2.56	0.85	0.45–0.50 (n = 124)	0.96	0.38–2.41	0.93
0.45–0.55 (n = 104)	2.67	1.08–6.62	0.033	0.50–0.55 (n = 104)	1.15	0.46–2.89	0.75
>0.55 (n = 57)	4.74	1.91–11.75	0.0008	>0.55 (n = 54)	Reference		
	Parameter estimate	Standard error	p-value		Parameter estimate	Standard error	p-value
Serum IgE (log transformed)[#]	0.69	0.52	0.19		−2.48	1.23	0.04
FVC[#] (L)	−0.46	0.28	0.11		0.49	0.53	0.36
FEV1[#] (L)	−0.27	0.24	0.28		−0.13	0.47	0.78
FEV1/FVC[#]	0.006	0.04	0.87		−0.15	0.08	0.06
FEF25-75%[#] (L/s)	−0.35	0.55	0.52		−1.35	1.04	0.19

[*] Statistically controlled for sex, gestational smoking, current smoking status, chest infection at one and two years of age, duration of breast feeding, farm exposure and number of days of exercise at age 18 years.
[#] Statistically controlled for sex, gestational smoking, duration of breast feeding (weeks), farm exposure, height at age 18 and number of days of exercise at 18 years of age, cell types.
[+] 95% confidence internals.





Samples	F1 at birth (Guthrie Cards)	Age 10	Age 18	Pregnancy	(20-25 years)
Female	25	34	245	121	

Fig. 2. Median Methylation of cg14472551 and cg01669161 at different ages in different subsamples and for five women with complete follow-up from Guthrie cards to pregnancy. In these subgroups, all participants at age 10 years have received tetanus vaccination. The following table represents the sample size at different ages represented in the graph. Cg14472551 showed lower methylation until age 10 further increased by age 18, followed by lower methylation during the pregnancy. It showed a low methylation in vaccinated individuals compared to the non-vaccinated individuals and during pregnancy. Cg01669161 showed a constant increased in the methylation over time with high methylation in vaccinated individuals and during pregnancy.

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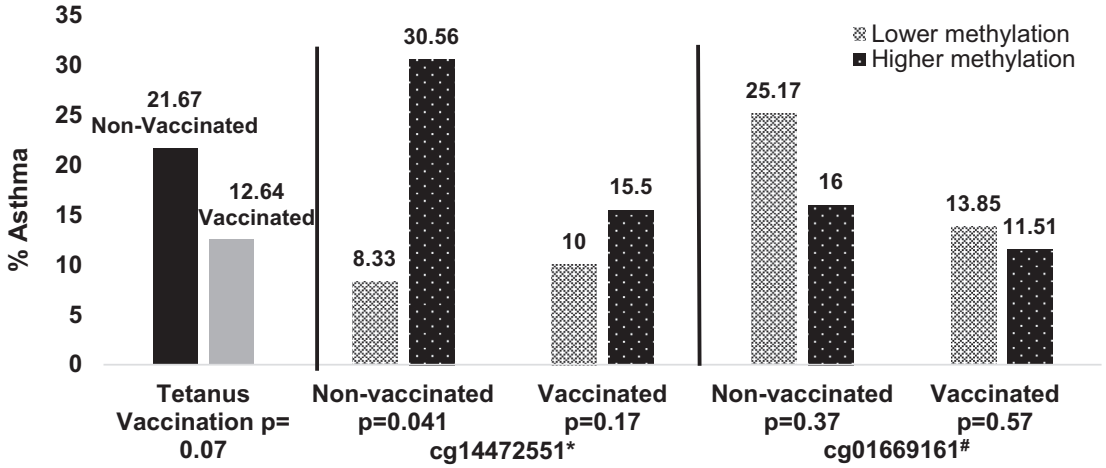

Fig. 3. The prevalence of asthma among vaccinated and non-vaccinated with lower and higher methylations individuals at 18 years of age. Higher and lower methylation groups were grouped based on the median methylation (β values) values. *ncg14472551 ⩽ 0.43 considered lower methylated and ncg14472551 > 0.43 as higher methylated. #ncg01669161 ⩽ 0.49 considered lower methylated and ncg01669161 > 0.49 as higher methylated. The prevalence of asthma is lower in vaccinated compared to non-vaccinated individuals. The prevalence of asthma is higher in non-vaccinated individuals whose cg14472551 methylation was higher while the prevalence was lower in vaccinated individuals. Individuals with higher methylation of cg01669161 has showed lower prevalence of asthma compared to lower methylation in both vaccinated and non-vaccinated individuals.

cal significance (Fig. 3). When following methylation over time (Fig. 2), we also observed that cg14472551 showed lower and cg01669161 higher methylation during pregnancy compared to other ages.

4. Discussion

Our study demonstrated that epigenetic changes due to vaccination can alter the risk of asthma at age 18 years. In an epigenome-wide screening we found that TVac was associated with differential DNA-M: decreased methylation of cg14472551 (*KIAA1549L gene*) and increased methylation of cg01669161 (intergenic region of *PSMG3* and *TFAMP1*) at age 18 years. We further investigated the association of these two CpGs with asthma and other allergic diseases. Following the effect of TVac on these two CpGs, both a higher and lower methylation of cg14472551 and cg01669161, respectively, were associated with an increased risk of asthma at 18 years of age. To our knowledge this is the first study to illustrate that TVac is associated with differential methylation which inturn has a protective effect on asthma in adolescence.

The locations of these two CpGs suggest a potentially functional connections to allergic disease. Cg14472551 is located upstream of the transcriptional start site of *KIAA1549L* (also known as *C11orf41*) on chromosome 11. The KIAA1549L protein is 1849 amino acids long and contains highly conserved regions [36,37]. *KIAA1549L* acts a fusion partner of *PAX5*, a transcription factor critical for B-cell commitment and maintenance [37]. From Gene Ontology annotations (http://www.uniport.org/uniport/Q6ZVL6) it was found that *KIAA1549L* is involved in the biological process of upregulation, stimulation, and activation of defense response to virus by the host. Since DNA-M and gene expression are inversely correlated a lower methylation on TSS may upregulate the expression of *KIAA1549L* and enhance host immunity.

Cg01669161 is located in the intergenic region (24,848 base pairs) between an antisense regulator of the proteasome assembly chaperone *PSMG3* and *TFAMP1* genes (a non-coding RNA) on chromosome 7. It is in close proximity (3000 base pairs downstream) of *PSMG3 (Proteasome assembly chaperon 3)*. Proteasomes are responsible for processing antigens for presentation on the cell surface, an essential step of vaccination-induced immunity. Close proximity of cg01669161 *PSMG3* may thus explain its role in TVac and allergy.

To further evaluate the functional role of these two CpGs, we investigated their association with lung function measurements and serum IgE. Serum IgE plays a key role in type I hypersensitivity (B-cell-induced IgE-mediated immune response) reactions such as allergy and asthma [38]. It also has an important role upstream of the cascade of reaction in inflammatory response in skin and lungs. Interestingly, higher methylation of cg01669161 is also associated with decreased serum IgE levels, which may potentially contribute to the protective effect against asthma.

For descriptive purposes, we also examined the differential methylation of these two CpGs at four age periods in female subgroups of the F1 generation using different (smaller) sample sizes. We observed that cg14472551 showed a higher methylation at birth followed by a decrease methylation over age while cg01669161 represented a lower methylation at birth followed by increased methylation over age. Cg14472551 was lower and cg01669161 was higher methylated during pregnancy and in vaccinated individuals, in agreement with the findings from the regression analyses. In five women with complete follow up the differential methylation of the two CpGs are in agreement with the cross-sectional findings of different samples.

After the identification of these two TVac-related CpGs, we continued the analyses to test whether these CpGs affect the prevalence of asthma and related markers. We noticed a decreased prevalence of asthma in vaccinated compared to non-vaccinated children (Fig. 3). In addition, cg01669161 was also significantly associated with decreased IgE levels, adding consistencies to our findings.

There are some limitations to this study. Since DNA-M measurements obtained using the Illumina Infinium HumanMethylation450 array have high reproducibility and validity [22,36], we did not technically replicate the DNA-M measurements. Second, methylation data were obtained from whole blood but not from specific cell subgroups, due to cost, but also because the differential methylation observed here may well be present in all cell subsets anyway. Nevertheless, since DNA-M of peripheral blood can be influenced by the cell composition, we controlled for the cell types in the EWAS. Third, in the descriptive part, we investigated DNA-M changes from birth to pregnancy. All individuals with complete follow-up were women, which restricted our observation of methylation changes over time to women (Fig. 2). Fourth, the small sample size at 10 years (n = 30) and the fact that nearly all

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participants (94.3%) were vaccinated at 10 years of age limited our comparison between vaccinated and non-vaccinated to the age of 18 year. Finally, since this is the first study that demonstrated an effect of vaccination on asthma that may be mediated via DNA-M and results in a reduced risk of asthma, further replication of these associations in an independent cohort is needed.

5. Conclusion

In an EWAS, we found that TVac was associated with differential methylation of two CpG site located in or near genes with functional connections to immunity and antigen presentation. These CpGs in turn were protective against asthma and increased IgE. This suggests that DNA-M act as a possible molecular mechanism of protective effects of TVac against asthma in adolescence. Vaccination and related methylation of specific CpGs may offer novel prospects to investigate and explain a differential occurrence of asthma in childhood/adolescence.

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Authors contributions to the study

VDJ analyzed data, tested hypotheses, and wrote the manuscript. GAL extracted DNA, managed acquisition of all methylation data, and revised the manuscript prior to submission. WK was involved in the design of the study, acquisition of the funding, hypothesis delineation, analyses of the data, and drafting and revision of the manuscript prior to submission. HZ was involved in hypothesis delineation, statistical advisement and in revisions of the manuscript prior to submission. JWH supervised the DNA-methylation measurement and revised the manuscript prior to submission. HA, FM, and RK were involved in the acquisition of the data and in revisions of the manuscript prior to submission. NM, FIR and RK were involved in drafting and revisions of the manuscript.

Conflicts of interests

The authors declare that they have no conflicts of interests.

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