

CORRESPONDENCE

Methylation of the filaggrin gene promoter does not affect gene expression and allergy

To the Editor,

Filaggrin (*FLG*) loss-of-function (LOF) mutations are associated with altered epithelial barrier function in affected individuals and with the development of eczema (1, 2). We have previously shown that *FLG* LOF mutations appear to play a role in the development of food allergy in the first year of life by increasing the risk of food sensitization (3). Food allergens are first encountered via the mucosal surfaces of the oral cavity. Therefore, if *FLG* is expressed in the mouth, individuals with *FLG* LOF mutations may have compromised barrier integrity in the oral cavity as well as the skin, which provides intriguing possibilities with regard to the development of food allergy.

DNA methylation is a well-characterized epigenetic mark associated with changes in gene expression. To address the potential for epigenetic regulation of *FLG* in the oral mucosa, we compared both *FLG* gene expression and *FLG* promoter region DNA methylation between matched skin and buccal cells from eight adult donors. As promoter methylation is typically associated with repression, we aimed to investigate methylation at proximal CpG sites near the transcription start site (TSS). Although the *FLG* promoter is CpG poor, containing only three CpG sites within 500-bp upstream of the TSS, several genes with similar CpG densities have previously been demonstrated to be regulated by DNA methylation, for example IL-2 (4) and IFN- γ (5). Of these three CpG sites, data from one CpG site (194-bp upstream of TSS) were subsequently excluded from the analysis due to the presence of a silent peak in the mass spectra. CpG sites 1 and 2 investigated are 409- and 532-bp upstream of *FLG* TSS, respectively (Figure S1 in the Supporting Information). We then examined the potential for novel associations between *FLG* DNA methylation and allergic outcomes in 158, 12-month-old infants from the HealthNuts study (6). See Supporting Information for Data S1 and Table S1 for assay primers and cycling conditions.

Relationship between *FLG* expression and promoter methylation in matched skin and buccal cells in adult donors

Using the formula $2^{-\Delta\Delta CT}$ (7), the normalized level of *FLG* expression in buccal samples relative to matched skin samples of adults was approximately 19% (Table 1), revealing evidence of (low level) expression of *FLG* in buccal cells. This exploratory study is the first to show that *FLG* is expressed in buccal cells. The lower expression in the buccal epithelia is logical because these cells are non-keratinized epithelia. There was little evidence of a correlation in *FLG* expression levels between the sample types ($R^2 = 0.06$).

Generally, the methylation levels at CpG sites 1 and 2 in skin were lower compared with buccal samples (Table S2). There was some correlation in *FLG* methylation levels between tissues within individuals at CpG site 1 ($R^2 = 0.32$) but not at CpG site 2 ($R^2 = 0.01$).

Within the eight adult individuals analysed, there was little evidence of negative correlation between *FLG* expression and methylation at CpG site 1 in skin ($R^2 = 0.13$) and no correlation in buccal samples ($R^2 = 0.00$). There was also little evidence of negative correlation between *FLG* expression and methylation at CpG site 2 in skin ($R^2 = 0.06$) and buccal samples ($R^2 = 0.34$).

Relationship between *FLG* DNA methylation and gene expression in infants

The overall average *FLG* methylation levels for infant buccal samples were comparable to that of the adult samples, with an average methylation β value of 0.81 ($n = 134$; range: 0.60–0.95) at CpG site 1 and an average $\beta = 0.98$ ($n = 138$) at CpG site 2, which was tightly regulated (range: 0.92–1.00). This relative consistency suggests that epigenetic regulation by means of DNA methylation, if any, is maintained the same way in infants and adults at these sites.

FLG expression was measured in 15 infant buccal cells. The average ΔCT was calculated as 3.68 ± 1.23 (Table S3). As matched skin samples were not available for infants, normalized *FLG* amount in infant buccal samples were calculated relative to adult buccal samples. $2^{-\Delta\Delta CT}$ was 2.06 (0.88–4.82), indicating that infant buccal samples have approximately twice the amount of *FLG* compared with adult buccal samples. This higher amount could suggest that other as yet unrecognized functions of filaggrin or its breakdown products might play a more important role in the infant mouth related to oral tolerance. There was a weak negative correlation between *FLG* methylation and expression at CpG site 1 in infant buccal samples ($R^2 = 0.08$).

FLG methylation in food-allergic infants

Within infant buccal samples, *FLG* methylation at CpG sites 1 and 2 was compared between those with and without eczema, food sensitization and food allergy (Table S4). A statistically significant lower average methylation level in the highly methylated CpG site 2 was noted in comparison with controls in those with a *FLG* mutation, those with eczema, and in peanut-sensitized tolerant infants. However, it is not clear whether a difference of 1%–2% methylation is likely to be either biologically or clinically relevant. Although *FLG* expression in infant buccal samples was lower in those with eczema

Table 1 Analysis of relative gene expression data in adult samples using the $2^{-\Delta\Delta C_T}$ method

Sample	<i>FLG</i> C_T (average/sample)	<i>GAPDH</i> C_T	ΔC_T (Avg. <i>FLG</i> C_T –Avg. <i>GAPDH</i> C_T)	$\Delta\Delta C_T$ (Avg. ΔC_T – Avg. ΔC_T , _{skin})	Normalized <i>FLG</i> amount relative to skin, $2^{-\Delta\Delta C_T}$
Skin 1	27.76	27.33	0.43		
Skin 2	29.76	26.76	3.00		
Skin 3	30.04	29.14	0.90		
Skin 4	30.50	27.43	3.06		
Skin 5	33.91	28.59	5.32		
Skin 6	35.31	33.30	2.01		
Skin 7	26.57	25.11	1.46		
Skin 8	30.70	28.30	2.40		
Average	30.57 $\pm$ 2.89	28.24 $\pm$ 2.39	2.32 $\pm$ 1.53	0.00 $\pm$ 1.53	1.00
Buccal 1	37.27	33.83	3.43		
Buccal 2	37.81	31.17	6.64		
Buccal 3	40.00	35.11	4.89		
Buccal 4	36.69	32.65	4.04		
Buccal 5*	–	–	–		
Buccal 6	34.50	30.19	4.30		
Buccal 7	39.27	33.09	6.18		
Buccal 8	40.00	36.39	3.61		
Average	37.93 $\pm$ 2.00	33.20 $\pm$ 2.15	4.73 $\pm$ 1.25	2.41 $\pm$ 1.25	0.19 (0.08–0.45)

The average cycle threshold (C_T) was calculated from triplicates of each sample, and ΔC_T was calculated by subtracting average glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (TaqMan[®], Applied Biosystem, Foster City, CA, USA) C_T from average *FLG* C_T in each buccal and skin sample. A $\Delta\Delta C_T$ value was calculated by subtracting the average ΔC_T of the skin (reference tissue) from the average ΔC_T of the buccal samples.

*Buccal sample 5 failed to be amplified in the qRT-PCR.

[mean ratio = 0.08 (95% CI, 0.04–0.12); $n = 10$] than those without eczema [mean ratio = 0.18 (95% CI, –0.10 to 0.52); $n = 5$], it was not statistically significant ($p = 0.124$). There was also a trend of decreasing *FLG* expression from negative control (NC) [mean ratio = 0.11 (95% CI, –0.55 to 0.78); $n = 2$] to food sensitized but tolerant (ST) participants [mean ratio = 0.09 (95% CI, –0.76 to 0.93); $n = 2$] down to food-sensitized and food-allergic (SA) participants [mean ratio = 0.08 (95% CI, 0.03–0.12); $n = 9$]. However, the differences between NC and ST ($p = 0.776$), between NC and SA ($p = 0.443$) and between ST and SA ($p = 0.846$) were not statistically significant. Lack's hypothesis states that epicutaneous exposure to food in early infancy increases the risk of food sensitization, but optimal oral exposure abrogates the development of food allergy amongst food-sensitized infants (8). Thus, further investigations into oral *FLG* expression using more samples may be worthwhile because association with food allergy-related phenotypes would lend biological support to this hypothesis. Our study limitation include sampling site of the oral mucosa was limited to the buccal mucosa (*FLG* may play a role in other areas of the mouth, e.g. hard palate) and small sample size, precluding firm conclusion.

Epigenetic marks can influence disease phenotypes through the interaction with specific DNA sequence variants (9). A study found that DNA methylation level at an intragenic CpG site (cg07548383) of *FLG* interacted with *FLG* mutations on the risk of eczema ($p = 0.02$) at 85% and 86% methylation levels (10). However, the mechanistic role of intragenic DNA methylation is not yet apparent, and the DNA used was extracted from whole blood, where *FLG* expression is unknown

and was not examined. We did not investigate this intragenic CpG site, but it will be interesting to do so in future. In our study, no interaction was found between *FLG* methylation levels and *FLG* mutations (R501X, 2282del4, R2447X, S3247X, 3702delG) in determining the allergic diseases investigated.

In conclusion, *FLG* was found to be expressed in the buccal cells of both adults and infants. However, little evidence was found to support a role for methylation at the CpG sites investigated in the regulation of *FLG* expression in buccal cells. No significant difference in *FLG* methylation and expression in buccal cells and various allergic outcomes suggest that the role of filaggrin in the oral cavity might not be important for oral tolerance.

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Conflict of interest

K.J.A. has received speaker's fees from Abbott, Alphapharm, Wyeth and Nutricia. The rest of the authors declare that they have no relevant conflicts of interest.

Ethical approval

Ethics approval was obtained from the Office for Children HREC (reference no. CDF/07/492), Department of Human Services HREC (reference no. 10/07) and Royal Children's Hospital HREC (reference no. 27047).

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Appendix 1

The HealthNuts investigators include Anne-Louise Ponsonby, Melissa Wake, Mimi L. K. Tang, Shyamali Dharmage, Lyle Gurrin, Melanie Matheson, Adrian Lowe, Nicholas Osborne, Pamela Martin, David Hill, Leone Thiele, Helen Czech, Deborah Anderson, Marnie Robinson, Dean Tey, Jeeva Sanjeevan, Giovanni Zurzolo.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Diagrammatic representation of the location of CpG site 1 and 2 in UCSC Genome Browser (GRCh37/hg19 Assembly).

Table S1. PCR primers for *FLG* methylation assay.

Table S2. Methylation data of CpG site 1 and 2 in adult skin and buccal samples.

Table S3. Analysis of gene expression data in infant buccal samples (n = 15).

Table S4. Difference in average methylation levels at CpG sites 1 and 2 by different allergic outcomes.

1 **Methylation of the filaggrin gene promoter does not affect gene**
2 **expression and allergy**

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23 **Methods:**

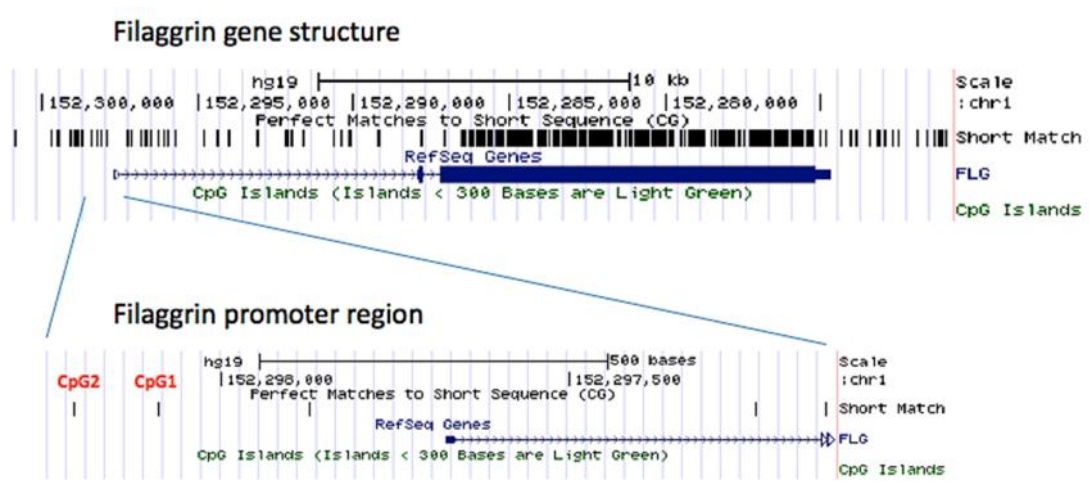
24 Skin samples from adult healthy donors were obtained by performing 3mm punch
25 biopsies on the inner arm while buccal samples (both adults and infants) were
26 collected using buccal swabs (Catch-All™ Sample Collection Swabs, Epicentre,
27 USA). DNA samples were extracted and bisulphite converted using MethylEasy™
28 Xceed (Human Genetics Signature, NSW, Australia) for adult samples and EZ-96
29 DNA Methylation-Gold™ (Zymo Research, Orange, CA) for high throughput
30 conversion of infant samples. RNA samples were extracted using TRIzol (Life
31 Technologies, US) and transcribed into cDNA using SuperScript™ III First-Strand
32 Synthesis System for RT-PCR (Life Technologies, US). All kits were used according
33 to manufacturer's protocol.

34 *FLG* expression was measured using predesigned TaqMan *FLG* qRT-PCR assay
35 (Hs00856927_g1, Applied Biosystem, USA) in LightCycler® 480 II (Roche,
36 Germany). DNA methylation levels at two CpG sites at the *FLG* promoter were
37 measured using the Sequenom MassARRAY EpiTYPER system (Sequenom, CA).

38 A student's t-test was used to analyse the difference in average *FLG* methylation
39 levels at two CpG sites and *FLG* expression in infants' buccal cells in association with
40 clinical outcomes of eczema, food sensitization and food allergy. *P*-value of <.05 was
41 considered statistically significant. T-tests were performed using Stata/IC 11.0 for
42 Windows (StataCorp, TX).

43

Figure E1: Diagrammatic representation of the location of CpG site 1 and 2 in UCSC Genome Browser (GRCh37/hg19 Assembly)



CpG site 1 genomic position: chr1:152,298,088-152,298,089

CpG site 2 genomic position: chr1:152,298,211-152,298,212

50 **Table E1:** PCR primers for *FLG* methylation assay

Direction	Primer*
Forward	AGGAAGAGAGGTTAATATAGGTTGTTTGTGTGTAGGGTTT
Reverse	CAGTAATACGACTCACTATAGGGAGAAGGCTCTCCTAAT AATCACCACCTTCTAATTCAATTA

51 *10 mer tag (5'-AGGAAGAGAG-3') was added to forward PCR primers while T7-promoter
52 tag and 8bp insert (5'-CAGTAATACGACTCACTATAGGGAGAAGGCT-3') was added to
53 reverse PCR primers. PCR was performed on bisulphite converted DNA using FastStart™
54 Taq DNA Polymerase (Roche) at the following thermalcycling conditions: 10 minutes initial
55 denaturation at 95°C; 5 cycles of 10 seconds denaturation at 95°C, 30 seconds annealing at
56 58°C, and 2 minutes extension at 72°C; 40 cycles of 10 seconds denaturation at 95°C, 30
57 seconds annealing at 62°C, and 1.5 minutes extension at 72°C; followed by 7 minutes final
58 extension at 72°C.

59 **Table E2:** Methylation data of CpG site 1 and 2 in adult skin and buccal samples

CpG site	Tissue type	Average methylation beta (β) value*	95% Confidence Interval	Range
1	Skin	0.63	0.58-0.68	0.54-0.70
	Buccal	0.80	0.77-0.83	0.71-0.85
2	Skin	0.90	0.88-0.92	0.87-0.93
	Buccal	0.96	0.94-0.98	0.92-0.98

60 *The average methylation level of each matched buccal and skin sample was calculated from
61 triplicates.

62 **Table E3:** Analysis of gene expression data in infant buccal samples (n=15)

Sample	Average <i>FLG</i> C _T	Average <i>GAPDH</i> C _T	ΔC_T (Avg. <i>FLG</i> C _T - <i>GAPDH</i> C _T)
3549	39.55	34.63	4.92
3707	37.22	32.70	4.53
3743	35.37	32.01	3.36
3839	39.17	36.02	3.14
4769	36.43	33.73	2.71
4899	39.81	35.79	4.02
4956	38.10	34.84	3.25
5035	39.51	36.92	2.59
5042	40.00	35.82	4.18
5136	37.67	33.59	4.09
5191	37.59	31.91	5.68
5204	38.44	33.32	5.12
5219	34.49	32.24	2.25
5318	36.91	35.89	1.03
5341	37.95	33.56	4.39
Average	37.88 ± 1.63	34.20 ± 1.63	3.68 ± 1.23

63 Using the average ΔC_T of 4.73 ± 1.25 from adult buccal samples, the $\Delta \Delta C_T$ for infant buccal
64 samples calculated was -1.04 ± 1.23 , resulting in normalised *FLG* amount in infant buccal
65 samples (n=15) relative to adult buccal samples (n=8), $2^{-\Delta \Delta C_T}$, of 2.06 (0.88-4.82).

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Table E4: Difference in average methylation levels at CpG sites 1 and 2 by different allergic outcomes

Group*	CpG site 1			CpG site 2		
	N	Mean	P-value	N	Mean	P-value
Eczema variables						
No eczema	31	0.82	0.128	31	0.99	0.048
Eczema†	44	0.80		46	0.98	
No eczema	48	0.81	0.231	49	0.98	0.487
Diagnosed eczema	33	0.80		35	0.98	
No eczema	72	0.81	0.401	75	0.98	0.200
Current eczema	18	0.80		18	0.97	
Food sensitization variables						
No sensitization	29	0.82	0.101	29	0.98	0.082
Any food sensitization‡	47	0.80		49	0.98	
No sensitization	29	0.82	0.084	29	0.98	0.249
Egg sensitization	38	0.79		39	0.98	
No sensitization	29	0.82	0.074	29	0.98	0.172
Peanut sensitization	23	0.79		24	0.98	
No sensitization	29	0.82	0.796	29	0.98	0.936
Sesame sensitization	5	0.81		5	0.98	
Food allergy variables§						
Negative control	29	0.82	0.154	29	0.98	0.246
Any food allergy	34	0.80		34	0.98	
Negative control	29	0.82	0.090	29	0.98	0.052
Food sensitized tolerant	2	0.74		2	0.96	
Food sensitized tolerant	2	0.74	0.071	2	0.96	0.192
Any food allergy	34	0.80		34	0.98	
Negative control	29	0.82	0.179	29	0.98	0.322
Egg allergy	32	0.80		32	0.98	
Negative control	29	0.82	0.186	29	0.98	0.318
Egg sensitized tolerant	4	0.78		4	0.98	
Egg sensitized tolerant	4	0.78	0.294	4	0.98	0.677
Egg allergy	32	0.80		32	0.98	
Negative control	29	0.82	0.260	29	0.98	0.983
Peanut allergy	10	0.79		10	0.98	
Negative control	29	0.82	0.111	29	0.98	0.039
Peanut sensitized tolerant	7	0.78		8	0.97	
Peanut sensitized tolerant	7	0.78	0.411	8	0.97	0.171
Peanut allergy	10	0.79		10	0.98	
Negative control	29	0.82	N/A	29	0.98	N/A
Sesame allergy	1	0.78		1	1.00	
Negative control	29	0.82	0.738	29	0.98	0.281
Sesame sensitized tolerant	3	0.83		3	0.97	
Sesame sensitized tolerant	3	0.83	N/A	3	0.97	N/A
Sesame allergy	1	0.78		1	1.00	
Negative control without diagnosed eczema	22	0.81	0.195	22	0.98	0.425
Any food allergy with diagnosed eczema	18	0.79		18	0.98	
FLG mutation status						
No FLG mutation	62	0.81	0.353	64	0.98	0.031
FLG mutation	6	0.79		6	0.97	

*All analyses were restricted to Caucasian infants age ≤ 1.5 years old.

†Eczema was defined as having parent-reported diagnosed eczema, itchy rash (not nappy rash) treated with steroids, and/or nurse-observed current eczema during recruitment.

‡Food sensitization was defined as an SPT response of 2 mm or larger or positive cap-*FEIA* results (>0.35 KU/L) to at least one of these 10 foods (egg, peanut, sesame, shrimp, cow's milk, cashew, almond, hazelnut, soy, and wheat).

§Food allergy was defined as having a positive oral food challenge outcome (>_3 concurrent noncontact hives/urticaria for >_5 minutes, angioedema, vomiting, and/or anaphylaxis) or a clearly objective and recent history of reaction (egg: <1 month; peanut and sesame: <2 months) to egg, peanut, and sesame or an SPT response of 8 mm or greater to shrimp, cow's milk, cashew, almond, hazelnut, soy, and/or wheat. Negative control subjects (nonsensitized and nonallergic) had SPT responses of 0 mm to all foods at recruitment and in the clinic and had negative oral food challenge outcomes to egg/peanut. Food sensitized tolerant subjects had food sensitization but were tolerant on oral food challenge to the sensitized food.

||*FLG* mutation was defined as having at least one mutation in R501X, 2282del4, R2447X, S3247X and/or 3702delG.