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ORIGINAL ARTICLE

Epigenetic programming underpins B-cell dysfunction in peanut and multi-food allergy

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Abstract

Objective. Rates of IgE-mediated food allergy (FA) have increased over the last few decades, and mounting evidence implicates disruption of epigenetic profiles in various immune cell types in FA development. Recent data implicate B-cell dysfunction in FA; however, few studies have examined epigenetic changes within these cells. **Methods.** We assessed epigenetic and transcriptomic profiles in purified B cells from adolescents with FA, comparing single-food-allergic (peanut only), multi-food-allergic (peanut and ≥1 other food) and non-allergic (control) individuals. Adolescents represent a phenotype of persistent and severe FA indicative of a common immune deviation. **Results.** We identified 144 differentially methylated probes (DMPs) and 116 differentially expressed genes (DEGs) that distinguish B cells of individuals with FA from controls, including differential methylation of the *PM20D1* promoter previously associated with allergic disorders. Subgroup comparisons found 729 DMPs specific to either single-food- or multi-food-allergic individuals, suggesting epigenetic distinctions between allergy groups. This included two regions with increased methylation near three S100 genes in multi-food-allergic individuals. Ontology results of DEGs specific to multi-food-allergic individuals revealed enrichment of terms associated with myeloid cell activation. Motif enrichment analysis of promoters associated with DMPs and DEGs showed differential enrichment for motifs recognised by transcription factors regulating B- and T-cell development, B-cell lineage determination and TGF-β signalling pathway between the multi-food-allergic and single-food-allergic groups. **Conclusion.** Our data highlight epigenetic changes in B cells associated with peanut allergy, distinguishing features of the epigenome between single-food- and multi-food-allergic individuals and revealing differential developmental pathways potentially underpinning these distinct phenotypes.

Keywords: B cells, epigenetics, multi-food allergy, peanut allergy, transcriptomics

INTRODUCTION

IgE-mediated food allergies (FAs) are a major group of allergic disorders with more than 90% of food-allergic reactions caused by egg, soy, cow's milk, peanuts, tree nuts, wheat, fish and shellfish.[1] While most egg and cow's milk allergies resolve within the first few years of life, only 22% of peanut allergy resolves by age 4, and it often persists into adulthood.[2,3] Moreover, 20–30% of peanut-allergic individuals are multi-food-allergic (MA), most commonly to tree nuts (up to 41.5% reported in an Australian adolescent cohort), which increases the risk of experiencing adverse allergic reactions.[4–10] Mechanisms leading to concurrent sensitisations to multiple allergens as opposed to a single allergen are currently unknown. As adolescents with food allergy (FA) represent a subset of the FA population that suffer from persistent FA and are at the highest risk of serious allergic reactions (with up to 35% of FA adolescents experiencing a severe allergic reaction over a year), understanding the immune drivers of persistent peanut and multi-food allergy remains a high priority research area.[4,10–12]

While the role of B cells as producers of IgE in FA is well documented, recent studies have reported diverse functional changes of B cells in FA, such as enhanced responses upon stimulation with TLR ligand CpG, as well as variations in tissue and circulating subpopulations.[13–18] Food-allergic children tend to show increased proportions of circulating B cells compared with non-allergic (NA) children, most apparently in naïve and memory (both switched and non-switched) B-cell populations.[14] Moreover, variations in regulatory B-cell (Breg) populations in allergic individuals provide evidence for the role of B cells in regulating the allergic response, with Breg-derived interleukin (IL)-10 inhibiting the release of Th2 cytokines.[17] We previously showed that B cells from children with egg allergy respond in a more inflammatory manner upon non-specific activation compared with non-allergic controls, characterised by a heightened production of IL-8, as well as impaired production of regulatory cytokines such as IL-10.[14] This suggests a multifaceted role of B cells in shaping the development and severity of allergic response in FA.

Food allergy is a complex phenotype, involving gene–environment interactions that are likely mediated by epigenetic mechanisms, such as DNA methylation.[19] Indeed, peripheral blood mononuclear cells (PBMCs) and purified T-cell populations from FA individuals show distinct DNA methylation patterns compared with NA individuals, affecting genes associated with T-cell activation, mTOR signalling and the MAPK pathway, with these effects more pronounced in persistent FA.[20–23] Despite mounting evidence of epigenetic variation in various immune cell types in association with FA and evidence of B-cell dysfunction in children with FA, epigenetic profiles of B cells in FA have been largely overlooked. In this study, we compared genome-wide DNA methylation and transcriptomic profiles in B cells of FA adolescents (10–14 years of age) between single-food-allergic (SA, peanut only) and multi-food-allergic (MA, peanut and at least one other food), and non-allergic (NA) individuals.

RESULTS

Differentially methylated probes associated with peanut allergy

DNA methylation profiling was carried out on purified B-cell genomic DNA using the widely validated Infinium MethylationEPIC arrays. A total of 26 individuals were included in this analysis: nine NA controls and 17 FA adolescents, consisting of both single-food (peanut)-allergic (SA, $n = 10$) and multi-food-allergic (MA, $n = 7$) individuals (Figure 1a, Table 1). Following quality control procedures, methylation data were available for a total of 770 475 probes, across all individuals, for downstream analysis. While differential methylation analysis did not find any probes following FDR correction, using an unadjusted $P < 0.05$ significance threshold, we identified 144 differentially methylated probes (DMPs) between NA and FA groups, with 77 probes showing higher methylation in FA (largest effect size of $\Delta\beta$ 0.36 and mean (SD) $\Delta\beta$ 0.14 ± 0.05) and 67 probes showing lower methylation in FA (largest effect size of $\Delta\beta$ 0.29 and mean 0.14 $\Delta\beta \pm 0.05$) (Figure 1b–d, Supplementary table 1). All DMPs were within

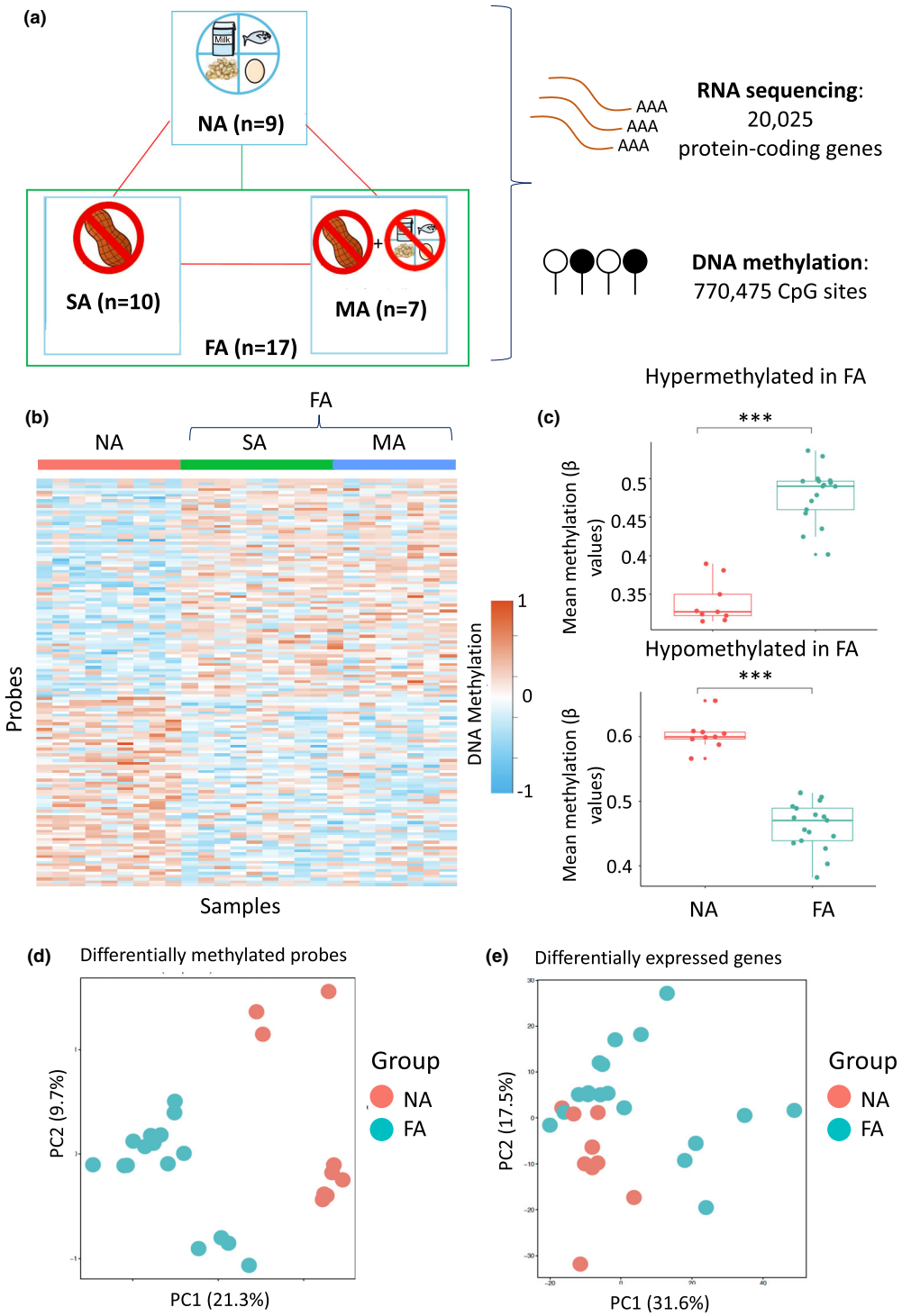

Figure 1. (a) Samples and groups [non-allergic (NA), n = 9; single-food allergic (SA), n = 10; and multi-food allergic (MA), n = 7] used in this study. Red lines indicate individual group comparisons carried out, while green lines represent the comparison of NA (n = 9) and combined FA groups (SA and MA, n = 17). 20 025 genes and 770 475 probes are used in transcriptomic and epigenomic analyses, respectively. **(b)** Heatmap of the 144 differentially methylated probes (DMPs) in the NA/FA comparison. **(c)** Boxplots of the (i) 77 probes showing higher methylation in FA individuals and (ii) 67 probes showing lower methylation in FA individuals compared with the NA group. Values displayed above boxplots represent *P*-values determined by the Mann–Whitney *U*-test between groups. **(d)** PCA plot of 144 DMPs separating the NA and FA groups. **(e)** PCA plot of 116 differentially expressed genes separating the NA and FA groups.

Table 1. Table of demographics for the subset of SchoolNuts cohort used in this study

	Non-food allergic (NA)	Peanut-only allergic (SA)	Multi-food allergic[a] (MA)
Total number	9	10	7
Sex: male, *n* (%)	4 (44)	5 (50)	5 (71)
Age at blood collection (years), median (min–max)	12 (10–14)	12 (11–13)	12 (10–13)
Current asthma/wheeze, *n* (%)	5 (56)	5 (50)	2 (29)
Hay fever ever, *n* (%)	6 (67)	6 (60)	6 (86)
Family history of food allergy, *n* (%)	5 (56)	2 (20)	2 (29) [1 ND]
Both parents born in Australia, *n* (%)	8 (89)	6 (60)	2 (29)
Peanut SPT (mm), median (min–max)	0 (0–2)	10 (6–22)	10 (3–22)
Peanut sIgE (kUA/L), median (min–max)	ND	9.27 (0.7–68)	18.1 (0.45–101)
Peanut allergy + ≥ one Tree nut allergy (%)	0 (0)	0 (0)	6 (86)

[a]Peanut allergy and allergy to one or more of the following foods: tree nuts (either cashew, pistachio, walnut, hazelnut, macadamia, pecan, almond, Brazil nut or pine nut), sesame, egg, milk or shellfish.

1 Mb of a gene transcription start site, with 15 genes in proximity to two or more DMPs. DMPs with higher methylation in association with FA were in the proximity of 59 genes, while DMPs with lower methylation in FA were associated with 56 genes.

Differentially methylated regions associated with peanut allergy

To further investigate methylation differences between NA and FA groups, DMRcate was used to identify differentially methylated regions (DMRs). A total of 288 DMRs were identified with a *P*-value < 0.05, and after applying further cut-offs (at least three probes per DMR and at least one of the probes with mean $\Delta\beta$ of 0.1), 17 DMRs remained (Figure 2a, Supplementary table 3). Of these, 10 showed higher (within 1Mb of *MAB21L3, FMOD, SPATA19, TTC23, GAREML, PIGZ, FAM53A, TGFBI, RNF39 and RARRES2*) and seven showed lower (within 1 Mb of *GSTM5, PM20D1, GNG13, SCN1A, ETS2, LPCAT1* and *PRKAG2*) methylation in association with FA. These 17 DMRs consisted of between 4 and 31 probes and ranged in size from 188 to 1517 base pairs.

This included a DMR in the promoter region of *PM20D1* (Figure 2b, c), previously identified in respiratory allergy (hay fever, rhinitis and wheezing) and atopic asthma,[24–26] consisting of 15 probes spanning 1531bp with lower mean DNA methylation in the FA group ($\Delta\beta$ 0.03-0.13, mean 0.08 $\Delta\beta \pm 0.04$). This included three probes identified as a statistically significant difference between NA and FA with a $\Delta\beta$ of > 0.1 (Figure 2b).

Single-food- and multi-food-allergic group-specific methylation signatures

Next, we aimed to identify methylation variation specific to the SA or MA groups. To do this, we compared the SA and MA with each other and with the NA group. Using previously defined cut-offs for the FA vs NA comparison (unadjusted *P*-value < 0.05 significance, $\Delta\beta > 0.1$), a total of 192 DMPs were identified between NA and SA groups (111 higher and 81 lower methylation); 335 between NA and MA (115 higher and 220 lower); and 318 between SA and MA (106 higher and 220 lower). A total of 729 unique DMPs (out of combined 845 DMPs) were identified across all comparisons (Figure 3a), of which all but three were within 1 Mb of 532 total genes (Supplementary table 4). A principal component analysis revealed distinct separation of the three groups based on methylation data at these 729 probes (Figure 3b), while unsupervised k-means clustering identified 12 distinct clusters of methylation variation, of which eight showed distinct methylation patterns across the three comparison groups (Figure 3c).

Differentially methylated regions associated with single-food- and multi-food-allergic groups

A total of 28 DMRs were identified between NA and SA groups (13 higher and 15 lower in SA, 217 total probes; consisting of between 4 and 22 probes, ranging in size from 188 to 2092 base pairs) within 1Mb of 28 genes. In contrast, 46 DMRs (400 total probes; consisting of between 4 and 32 probes, ranging in size from 142 to 2937

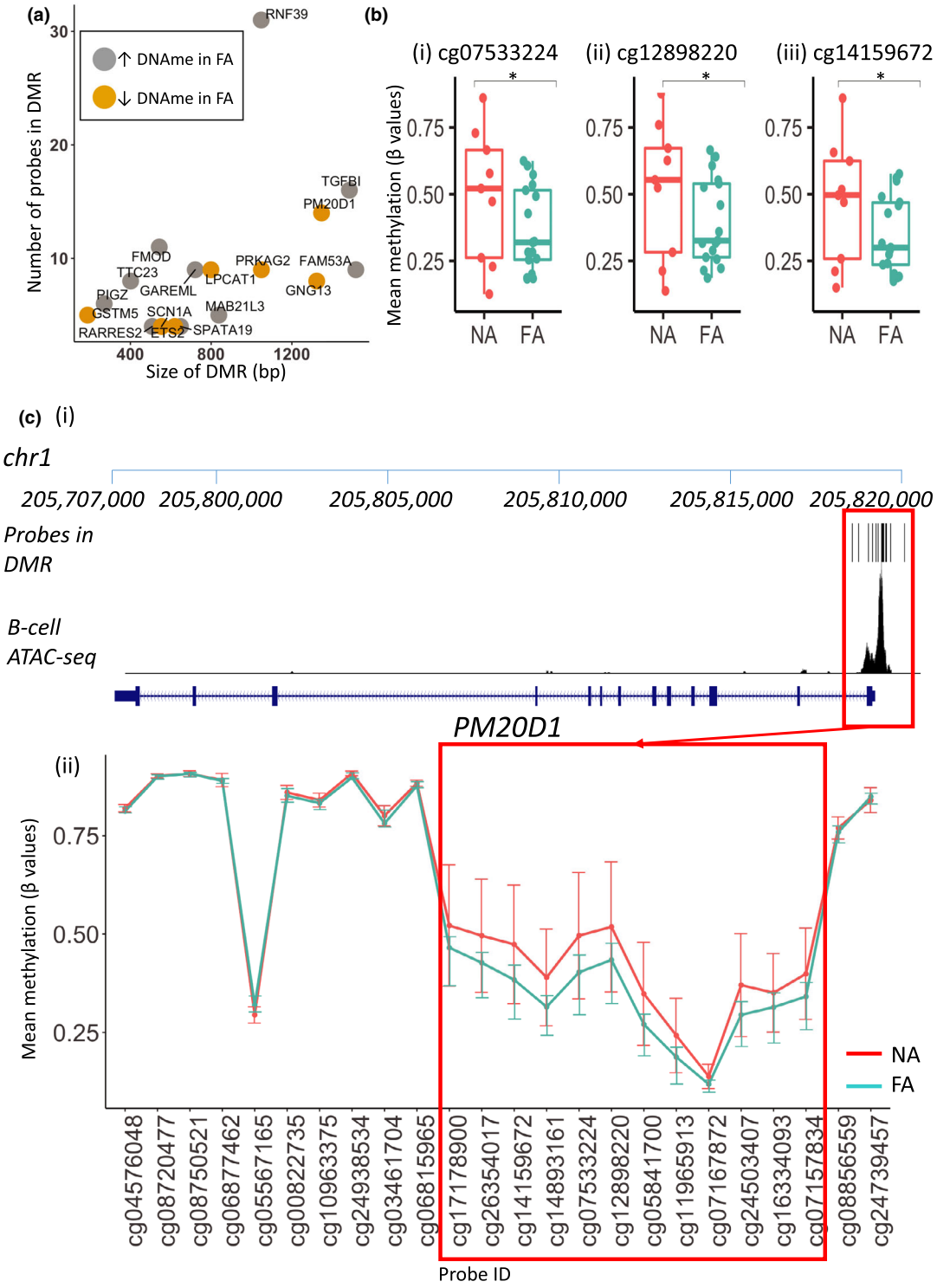

Figure 2. (a) Analysis of differentially methylated regions (DMRs) using DMRCate revealed 17 DMRs between NA (n = 9) and FA groups (n = 18). Here, we show a plot of DMR size (in base pairs (bp)) and number of probes in DMR. **(b)** A DMR associated with the *PM20D1* gene was found in this analysis, consisting of 15 probes. Displayed here are boxplots of statistically significant probes (as determined using the linear regression model with limma) with highest methylation difference between FA and NA groups in *PM20D1* DMR. Values displayed above boxplots represent *P*-values determined using the linear regression model (limma) **(c)** Location of the *PM20D1* DMR relative to *PM20D1* gene. (i) Location of DMR probes on the chr1 chromosome (p arm) and proximity to nearest gene, *PM20D1*. Overlaid with ATAC-seq data from healthy B cells to indicate regions of open chromatin. (ii) Mean methylation values (β values) of probes associated with *PM20D1*.

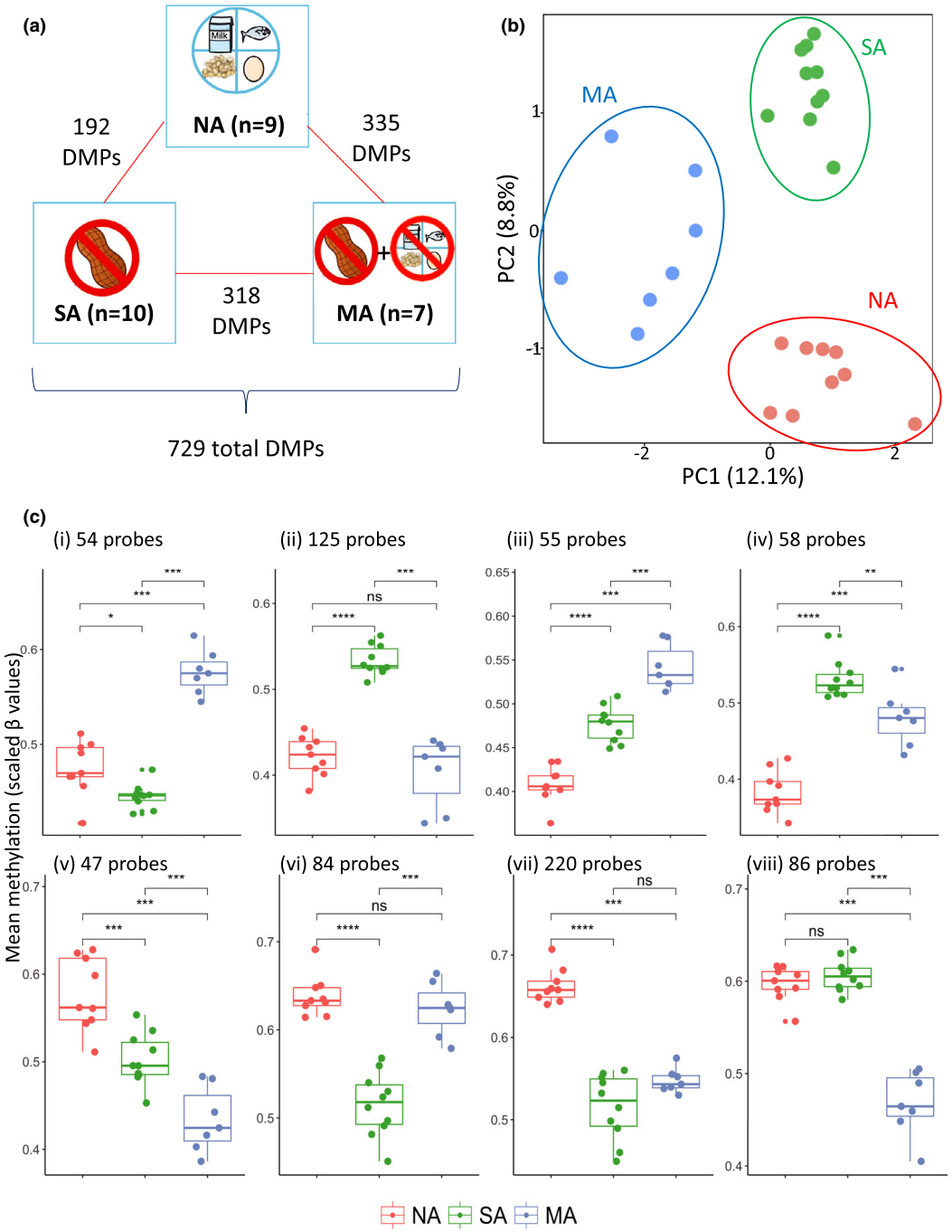

Figure 3. **(a)** Summary of the numbers of differentially methylated probes found in each group comparison (NA vs SA – 192 DMPs; NA vs MA – 335 DMPs; and SA vs MA – 318 DMPs), amounting to a total of 729 DMPs across all these comparisons. **(b)** PCA plot of these 729 probes from combined group comparisons showing distinct separation of the three clinical groups (NA in red, SA in green and MA in blue). **(c)** Unsupervised clustering of these 729 probes revealed 8 clusters. Shown here are boxplots comparing mean methylation values (β values) of samples in each group (NA, SA and MA) in these eight clusters and numbers of probes in each cluster. Values displayed above boxplots represent *P*-values determined by the Mann–Whitney *U*-test between groups. The 8 clusters were classified as follows: cluster (i) 54 probes progressively hypermethylated in allergic groups (methylation level: NA< MA< SA), (ii) 125 probes progressively hypermethylated in allergic groups (NA< SA< MA), (iii) 55 probes progressively hypomethylated in allergic groups (NA>MA>SA), (iv) 58 probes hypomethylated in allergic groups (NA>SA>MA), (v) 47 probes specifically hypermethylated in SA (NA = MA< SA), (vi) 84 probes specifically hypomethylated in SA (NA = MA>SA), (vii) 220 probes specifically hypermethylated in MA (NA = SA< MA) and (viii) 86 probes specifically hypomethylated in MA (NA = SA>MA).

base pairs) were identified between NA and MA (34 higher and 12 lower in MA) within 1Mb of 44 genes (Figure 4a), while 34 DMRs (250 total probes; consisting of between 4 and 14 probes, ranging in size from 183 to 1913 base pairs) were found between SA and MA (21 higher and 13 lower in MA), within 1Mb of 34 genes (Figure 4a). In total, the 95 DMRs identified across all comparisons covered 759 probes within 1Mb of 93 genes (Supplementary table 5).

Interestingly, two DMRs, spanning 6 and 22 probes, respectively, were found in close proximity within a cluster of *S100A1, S100A13* and *S100A14* genes on chromosome 1 (Figure 4b). Both DMRs exhibited higher methylation in the MA group relative to SA or NA groups ($\Delta\beta > 0.1$), with 10/28 probes in these DMRs showing significant methylation differences (Figure 4c). Further analysis of this region using publicly available ENCODE data revealed that these DMRs are marked by H3K27ac (a marker of active chromatin) and specific ATAC peaks (open chromatin associated with gene activity) in purified B cells[27] (Figure 4b). We also identified a DMR showing hypermethylation in the MA group (spanning 329 bp and consisting of six probes, two of which show 0.1 $\Delta\beta$ in the SA/MA comparison) near *RPTOR*, a gene for which we previously reported an unrelated DMR.[28] The DMR found in this study was located 200 Kb downstream of the TSS (within the gene body) and is annotated as an ENCODE enhancer site (Supplementary table 5).

Distribution of differentially methylated probes and regions

In order to determine the relevance of our findings, the distribution of the DMPs and DMRs from these comparisons relative to CpG islands was assessed. Relative to the overall distribution of EPIC probes relative to CpG islands, we did not find any particular enrichment for the distribution of DMPs from these comparisons in any locations. However, DMRs from these comparisons were increasingly located at the shores of CpG islands (Supplementary figure 3a).

To further assess whether these genes are actively transcribed in B cells, we used a publicly available data set of ATAC-seq data from healthy B cells.[27] Across all EPIC probes, only 23% (201 368/866 836) of probes are associated with B-cell-specific open chromatin regions. In contrast, 38% (55/144) of DMPs distinguishing between NA and FA individuals, and 31% (225/729) of DMPs distinguishing between the three clinical groups (NA, SA and MA) were associated with open chromatin regions in B cells (Supplementary figure 3b). Moreover, 47% (8/17) and 59% (56/95) of DMRs from these respective comparisons showed ATAC-seq peaks in healthy B cells (Supplementary tables 1, 3–5). A chi-square test reveals a statistically significant (chi-square statistic = 31.1899, *P*-value < 0.00001) enrichment for accessible chromatin regions among these DMPs and DMRs, indicating active transcription and therefore functional relevance of these sites in B cells.

Transcription factor (TF)-binding motif analysis of the open chromatin regions associated with these DMPs and DMRs reveals enrichment for 85 motifs, for which 40 associated TFs were expressed in our data (RPKM > 1). Among these, we found several binding sites recognised by TFs associated with B-cell lineage determination (IRF8, EBF1, PU.1, KLF3 and PU.1:IRF8) (Supplementary figure 4, Supplementary table 10).[29–34]

B-cell transcriptomic differences associated with peanut allergy

RNA profiling was carried out on purified B-cell mRNA using the next-generation platform, Illumina NovaSeq 6000. A total of 26 individuals were included: nine NA controls and 17 FA adolescents, consisting of both single-food (peanut)-allergic (SA, $n = 10$) and multi-food-allergic (MA, $n = 8$) individuals (Figure 1a, Table 1). Differential gene expression analysis revealed a total of 116 differentially expressed genes (DEGs; 23 upregulated and 93 downregulated in FA) with mean RPKM > 1, fold change of 1.5 and an unadjusted *P*-value < 0.05 between NA and FA groups (Supplementary figure 5). This revealed differential expression of genes associated with epigenetic and transcriptomic regulation (*HIST1H1D* and *PRDM15*) and cell differentiation and proliferation (*TIMP1*).[35,36] A principal component analysis of data for DEGs revealed some separation of NA and FA groups (Figure 1e, Supplementary table 2), but not to the same degree as the 144 DMPs (Figure 1d). The PCA indicates an overlap of a few NA and FA individuals who do not appear to share any similar traits (Figure 1e). Functional enrichment analysis did not identify any relevant

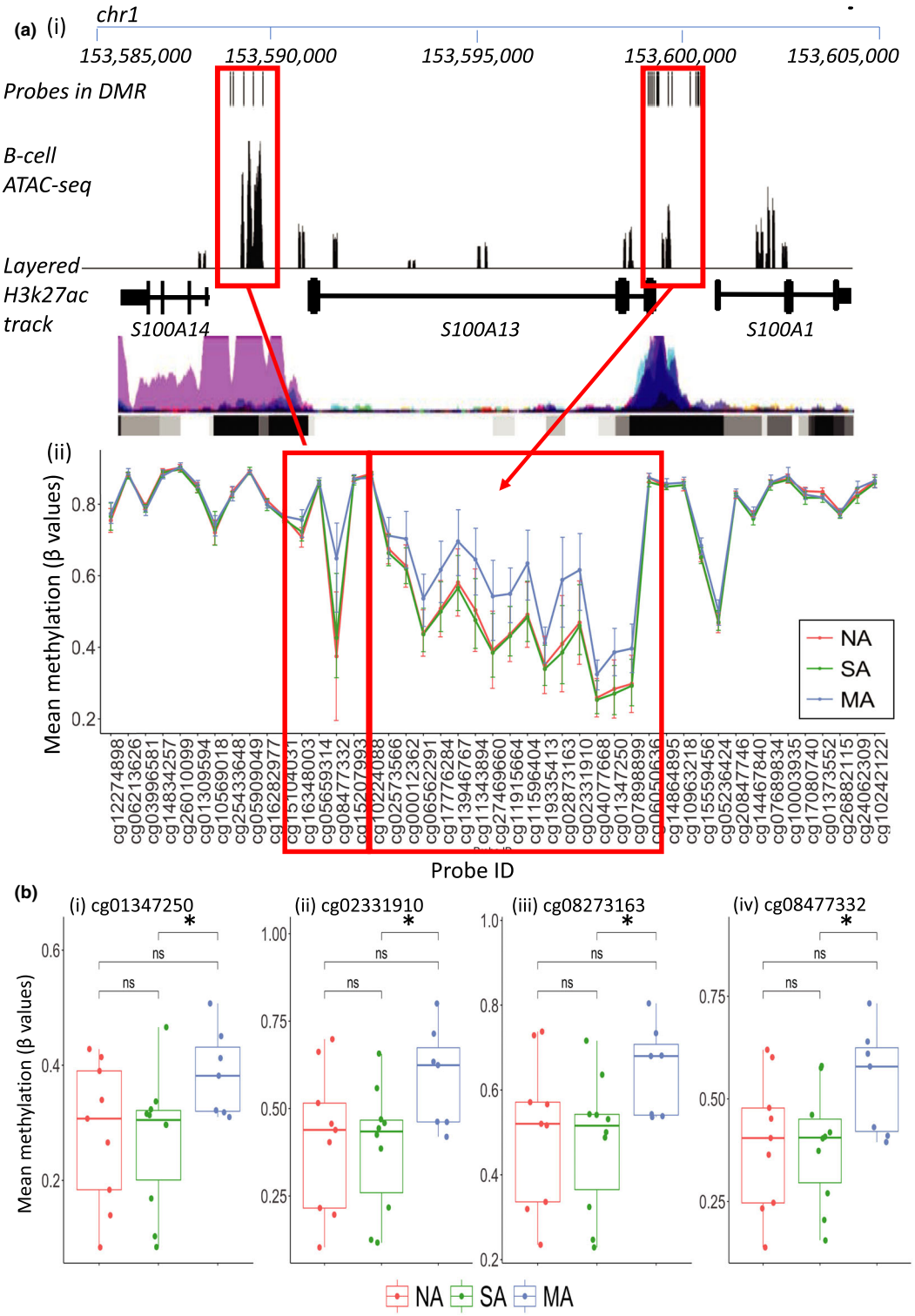

Figure 4. (a) Location of DMRs associated with S100 genes relative to gene locations on chromosome. (i) Location of probes on the chr1 chromosome (p arm) and proximity to nearest genes: *S100A14, S100A13* and *S100A1*. Overlaid with ATAC-seq data from healthy B cells to indicate regions of open chromatin, and layered H3K27ac track. (ii) Mean methylation (β values) of probes in this region across groups. **(b)** Boxplots indicating methylation values (β values) of selected probes within DMRs associated with S100 genes (i) cg01347250, (ii) cg02331910, (iii) cg08273163 and (iv) cg08477332. Values displayed above boxplots represent *P*-values determined by the Mann–Whitney *U*-test between groups.

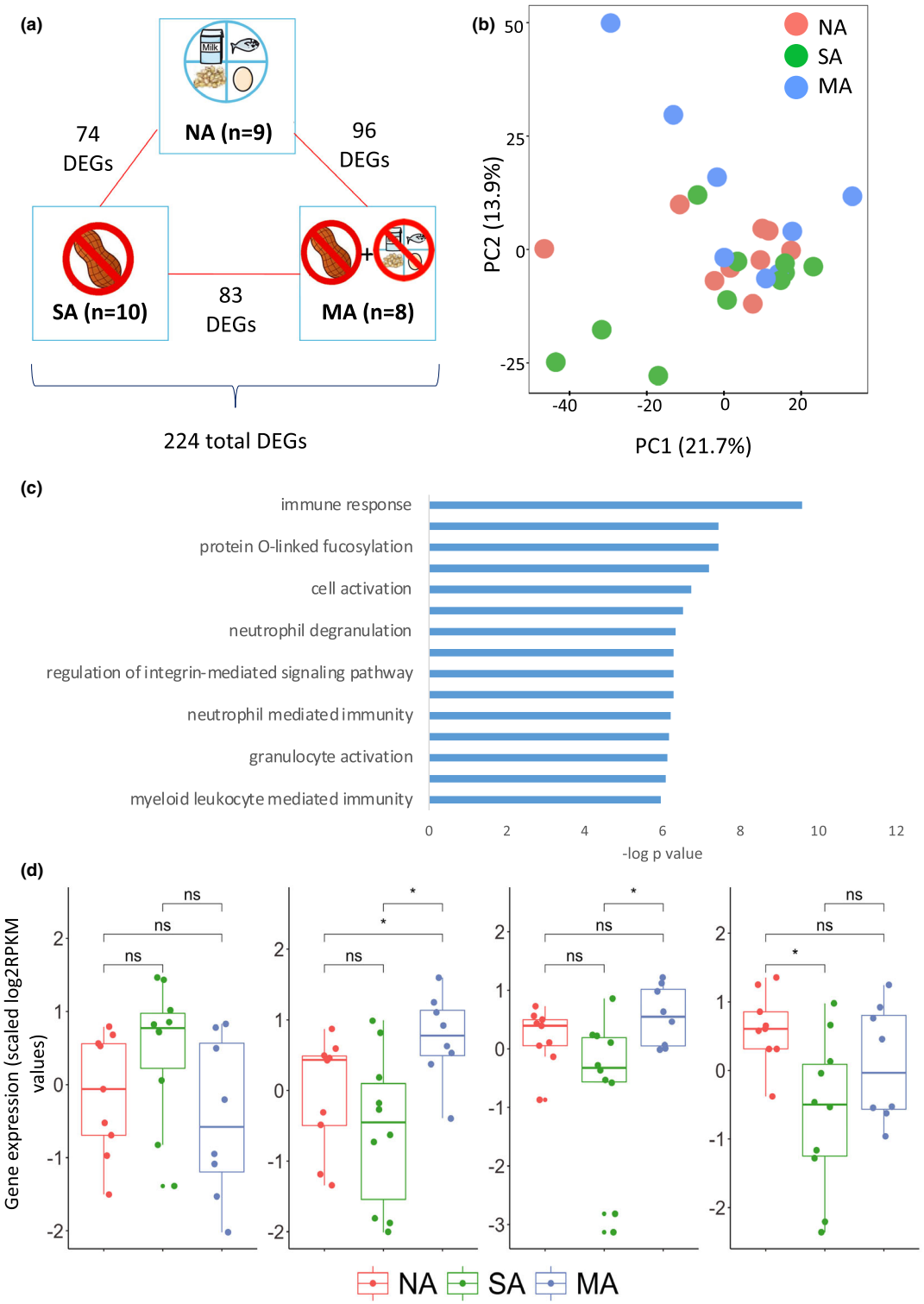

Figure 5. **(a)** Summary of numbers of differentially expressed genes (DEGs) found in each group comparison (NA vs SA – 74 DEGs; NA vs MA – 96 DEGs; and SA vs MA – 83 DEGs) and total numbers of DEGs (224 genes) across all comparisons. **(b)** PCA plot of the 224 DEGs across all groups. **(c)** Results of functional enrichment analysis of genes in the NA vs MA comparison, GO term visualised alongside -log *P*-value of associated term. **(d)** Boxplots indicating expression (scaled log2 RPKM values) of selected genes from the 224 DEGs identified across all groups: (i) *TNFRSF17*, (ii) *ANXA1*, (iii) *IL3RA* and (iv) *LCP2*. Values displayed above boxplots represent *P*-values determined by the Mann–Whitney *U*-test between groups.

gene ontology terms based on these 116 genes. However, enrichment analysis in the MSigDB curated gene set database revealed enrichment for genes previously implicated in T-cell activation, specifically regulatory T cells and Th2 cells, as well as mast cell activation[37–40] (Supplementary figure 6a).

Single-food- and multi-food-allergic group-specific B-cell transcription signatures

To identify group-specific gene expression patterns, we compared the SA and MA with each other and with the NA group, as previously done for DNA methylation. This revealed a total of 74 DEGs between NA and SA (33 upregulated in SA and 41 downregulated), 96 DEGs between NA and MA (60 upregulated 36 downregulated in MA) and 83 DEGs between SA and MA (51 upregulated and 32 downregulated in MA) (Figure 5a). Combined together, there were a total of 224 DEGs across these comparisons. However, as with the NA/FA comparison, a high degree of variability of gene expression existed within groups, with only a few genes showing consistent expression patterns across groups (Supplementary table 6). A principal component analysis of these genes does not indicate a clear distinction of the two allergic groups (Figure 5b).

Among these 224 DEGs, we found that *TNFRSF17* is upregulated specifically in the SA group, while *LCP2* is downregulated in the SA group. Conversely, *ANXA1* and *IL3RA* are upregulated in the MA group (Figure 5d). *TNFRSF17* encodes the B-cell maturation antigen (BCMA), which is responsible for the regulation of B-cell development and highly expressed by long-lived plasma cells, while *LCP2* plays a key role in immune cell signalling.[41,42] *ANXA1* serves a number of roles in the innate immune pathway, most notably as a regulator of inflammation.[43]

Gene ontology analysis of genes from the NA vs MA comparison revealed functional enrichment for the biological process immune response (GO:0006955) with nearly 20 genes from the target list populating this term (Figure 5c). Further investigation of the results indicates that these genes are more specifically involved in immune pathways such as myeloid leucocyte activation (GO:0002274), which included granulocyte activation (GO:0036230).

Transcription factor-binding motif analysis of genes in the proximity of differential methylation

In order to identify potential upstream regulators of the observed DNA methylation variation identified in specific allergy groups, we analysed distal and proximal regulatory regions for transcription factor-binding motifs. A total of 30 motifs were enriched across all comparisons in the distal regulatory regions, and of these, evidence of expression of the associated transcription factors was found for 11 of 30 motifs (mean RPKM $>$ 1) (Supplementary figure 6b, Supplementary table 8).

Interestingly, sites associated with lower methylation in MA showed depleted motif presence for RUNX1 binding, directly associated with the regulation of T-cell development[44] and enrichment (increased motif presence) of the motif recognised by b-MYB, a key regulator of cell differentiation, and FLI1 and ETS1, which play crucial roles in the activation of B-cell receptors.[45,46] In contrast, DMPs showing higher methylation in MA were enriched for PU.1 binding, implicated in B-cell maturation.[47]

In the proximal regulatory regions, 79 enriched motifs were identified, with evidence of expression of associated transcription factors for 32 of 79 motifs (Supplementary table 7). As with motifs mapping to distal regulatory sites, differential enrichment was observed in TF-binding sites associated with the development and maintenance of B- and T-cell populations (Figure 6b). Binding sites for Bcl6, PPAR-α, Zfx, OCT2 and NFAT[48–56] showed depleted enrichment, whereas motifs associated with B-cell lineage determination (PU.1, IRF4, KLF3, Bcl11a and PU.1:IRF8) were enriched in the MA group.[29–33] We additionally found depleted motif presence for regulators of the B-cell receptor (SpiB and PU.1) in MA groups.[47,57,58] Interestingly, binding sites for TFs regulating the TGF-β signalling pathway were only enriched in the promoters associated with DMPs showing higher methylation in MA (SMAD2 and SMAD4).[57]

Transcription factor-binding motif analysis of differentially expressed genes

Motif enrichment analysis of DEG-associated promoters revealed 31 enriched motifs (with evidence of expression for 13 of 31 genes

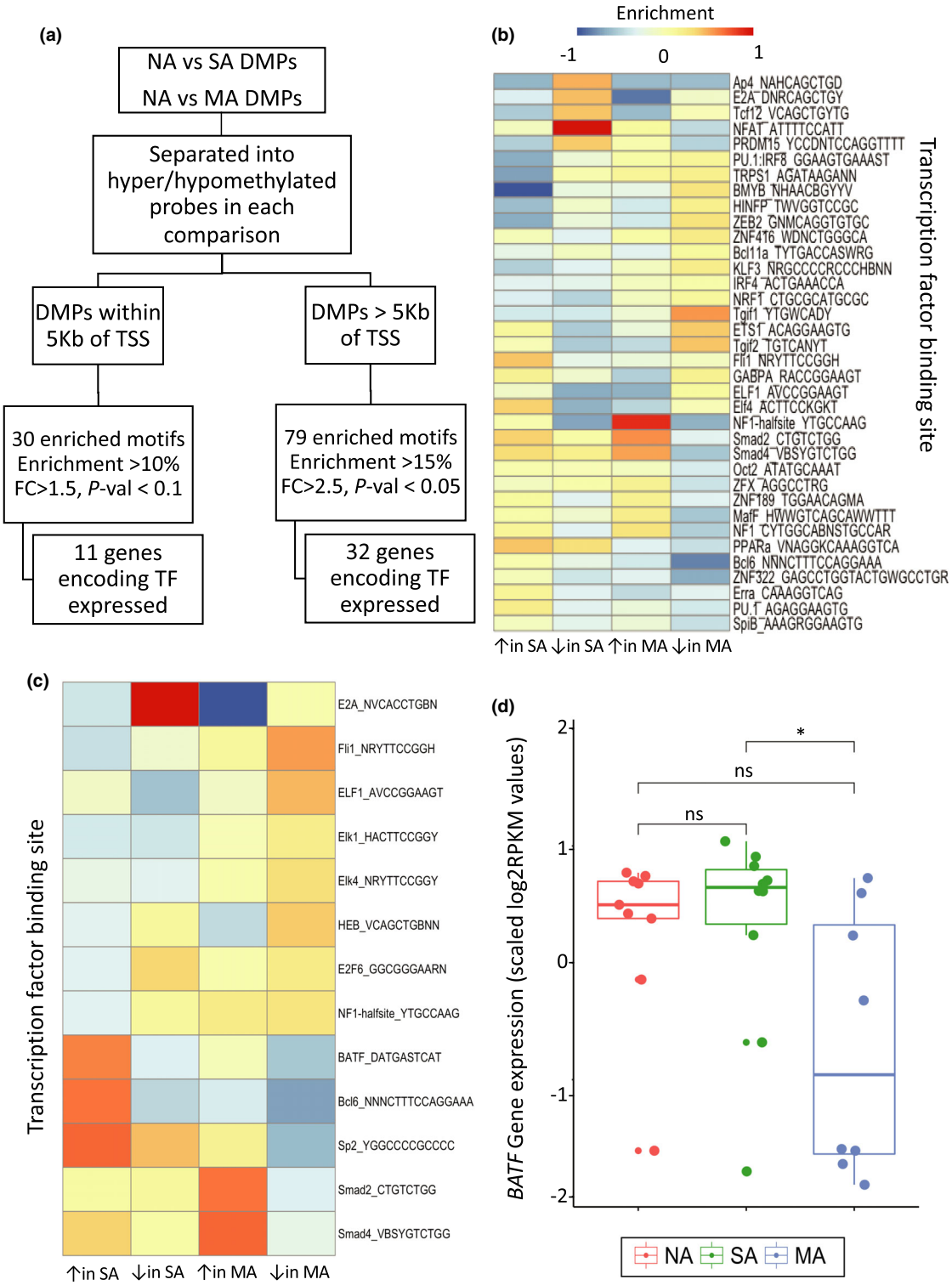

Figure 6. (a) Workflow employed to carry out motif enrichment analysis. **(b)** Heatmap showing enrichment of motifs across promoters associated with DMPs from NA vs SA and NA vs MA comparisons. **(c)** Heatmap showing enrichment of motifs in promoters associated with DEGs from NA vs SA and NA vs MA comparisons. **(d)** Expression (scaled log2 RPKM values) of *BATF* gene across groups.

encoding transcription factors that recognise these motifs) across the NA/SA and NA/MA comparisons (Figure 6c, Supplementary table 9). Consistent with the motif signatures of DMP-associated promoters, binding sites for Bcl6 and BATF, involved in the development and maintenance of B- and T-cell populations, showed depleted enrichment in MA-associated DEGs.[29,58] Interestingly, *BATF* gene expression was also downregulated in the MA group (Figure 6d). Moreover, we found that promoters associated with upregulated genes in MA showed enrichment for SMAD2 and SMAD4.

To further determine the significance of TGF-β pathway in our data, the expression of genes associated with the TGF-β pathway was assessed. Overall, 42 of 86 genes contained in the TGF-β pathway KEGG gene set were expressed (RPKM > 0) across all samples. However, SA individuals exhibit diminished expression of these genes, suggesting dysregulation of the TGF-β pathway in peanut-only-allergic individuals (Supplementary figure 5c).

Distinct methylation and gene expression signatures in B cells of FA individuals

To assess the degree to which differential methylation is associated with differential gene expression in B cells of FA individuals, we directly compared genes in the proximity of DMPs/DMRs with DEG. This revealed minimal overlap, with only 11 of 224 total DEGs having a DMP near them, and no DEGs with a nearby DMR. This low overlap between DMPs and DEGs is also reflected in the generally different motif signatures, except for enrichment of Bcl6 and BATF, suggesting that these transcription factors may be involved in the differential regulation of B cells in FA.

DISCUSSION

This is the first analysis of combined genome-wide DNA methylation and transcriptomic profiling of purified B cells from peanut-allergic individuals and the first to assess molecular profiles of single- and multi-food-allergic adolescents, an understudied yet important population given the complex health consequences of FA in this age group.[59,60] Our findings are consistent with past studies in other cell types showing epigenetic variation in association with FA,[21,28,61] but we extend this by revealing distinct epigenetic variation specific to the MA phenotype.

In summary, DNA and RNA samples from 26 individuals were from three clinical groups: patients with single-food allergy (peanut only), patients with multi-food allergy (peanut and one or more additional foods) and non-allergic controls. Comparisons of FA and NA were carried out with FA groups in combination and individually, and we found group-specific signatures, highlighted by increased numbers of DEGs/DMPs when FA groups were analysed separately.

B cells from peanut-allergic individuals show widespread differential methylation

Whereas previous analyses have examined epigenetic variation associated with PBMCs and T cells from FA infants, our findings support the emerging concept that molecular pathways are disrupted across multiple cell types in FA, possibly indicative of early-life reprogramming of cells that persists into adolescence.[62] Indeed, a recent study carried out on the SchoolNuts cohort, the same cohort of adolescents used in this study, found that adolescents with FA possess unique immune profiles that reflect changes reported in early life, such as increased proportions of dendritic cells and activated regulatory T cells in FA individuals.[28,62] Such differences may be attributed to the unique epigenetic signature of FA adolescents found in this study. Our finding of the DMR associated with *PM20D1*, a gene encoding peptidase M20 domain-containing one enzyme, supports this hypothesis. Higher methylation at this locus has been strongly associated with adult obesity (up to 0.2 $\Delta\beta$) and moderately with history of stroke (0.05 $\Delta\beta$), suggesting that this particular DMR is associated with a number of phenotypes,[63,64] potentially those associated with inflammation. In a study by Gunawardhana *et al*. (2014), DNA methylation profiled in peripheral blood samples of 12-month infants (n = 40) showed lower methylation (0.1 $\Delta\beta$) at this locus in individuals born to atopic mothers.[25] While there was no assessment of atopic status of offspring, subsequent studies in later life reported higher methylation at this region in association with respiratory allergy (IgE sensitisation to a set of airborne allergens, in addition to doctor's report of hay fever or rhinitis) and wheezing.[24,26] Despite the differences in

previous studies, it is clear that this represents a region subject to epigenetic variation across the life course including a range of allergic disorders, namely respiratory allergy and now FA. *PM20D1* is known to be a regulator of *N*-fatty acyl-amino acids, which are involved in a number of endogenous signalling pathways that include regulation of inflammation. The methylation status of *PM20D1* may therefore have downstream effects on allergy-related inflammatory responses.[65]

B cells of single- and multi-food allergies appear distinct at the molecular level

Our findings also indicate that MA individuals are epigenetically distinct from SA individuals, clearly illustrated in the group-specific methylation signatures in the probes. Hypermethylation of regions near genes encoding S100 proteins indicates differential regulation of these pathways associated with these proteins in MA individuals. The S100 family encompasses 21 calcium-binding proteins that serve diverse roles in inflammation, energy metabolism and cell cycle regulation.[66–68] *S100A1* and *S100A13* have been previously linked to allergy in a study documenting the suppressive effects of anti-allergic drugs (cromolyn, amlexanox and tranilast, used to suppress the inflammatory response) on these proteins.[69,70] In a study of atopic dermatitis, *S100A11* expression was found to be modulated by Th2 cytokines, which have a downstream effect on human β-defensin and filaggrin response, both of which have been associated with the progression of allergic disorders.[71] Enrichment for accessible chromatin in healthy B cells across the DMPs and DMRs found across these comparisons provides evidence for transcription factor activity at these regions. This may provide some insight regarding pathways contributing to the development of multi-food allergy.

Limited concordance between B-cell gene expression and differential methylation in FA

While our results indicate differences in gene expression between NA and FA B cells at baseline, expression levels were variable across groups and the findings on transcriptomic differences did not correlate well with the extensive epigenetic differences observed. However, differences in expression of key genes involved in immune cell signalling and development (*LCP2* and *TNFRSF17*), cytokine activity (*IL3RA*) and regulation of inflammation (*ANXA1*) across groups support our hypothesis that crucial molecular pathways are disrupted in FA.[41–43,72] Functional enrichment for terms associated with immune responses in MA-associated DEGs, in particular granulocyte activation, suggests that B cells mediate myeloid cell activity and potentially prompt an inflammatory phenotype in MA individuals even at baseline. While *ANXA1* is largely associated with the anti-inflammatory response, one study reports increased expression of *ANXA1* in plasma samples ($n = 100$) of asthmatic patients compared with controls.[43,73,74] This suggests that the production of higher *ANXA1* levels at baseline counteract the proinflammatory skew in atopic individuals. Despite moderate gene expression differences found in this study, studies from our group have demonstrated increased proinflammatory responses of B cells from FA samples following activation via cytokine assays, indicating that B-cell function is extensively altered in FA individuals.[14] Future studies should investigate differences in gene expression of B cells following activation in an *in vitro* model of allergen exposure to provide insight into the pathways underlying this altered response, and characterise differential responses to activation in MA individuals.

B-cell lineage-specific motif signatures identified in DMP- and DEG-associated promoters

The results of our motif analysis shed some light on how B cells interact with other cells to develop the adverse immune responses observed in allergic adolescents. Notably, the differential enrichment for motifs binding OCT2, NFAT, BATF and Bcl6 indicates differential pathways regulating B- and T-cell development in MA individuals, with the differential expression of BATF providing further evidence for disruptions in immune cell development in MA patients.[48–50,52–55] Enrichment of Bcl6 motifs has previously been associated with chronic inflammatory disorders such as juvenile arthritis,[76] and previous studies report the combined roles of OCT2 and NFAT in promoting a proinflammatory response to pathogens in B- and T-cell populations via the production of IL-6 and IL-21.[48,49] Differential enrichment of binding sites

for TFs determining B-cell lineage (KLF3, Bcl11a and PU.1:IRF8) and regulating B-cell receptor signalling pathways (SpiB and Pu.1) suggests that MA individuals may possess unique B-cell subpopulations and exhibit differential activation responses compared with SA individuals.[14] This is further supported by the differential enrichment of PPAR-α between MA and SA groups shown by our data, which has been associated with the development of regulatory B cells.[56] Moreover, differential enrichment of SMAD2 and SMAD4 promoters between MA- and SA-associated DEGs and DMPs implicates the TGF-β signalling pathway in the FA phenotype. The TGF-β signalling pathway plays a key role in the regulation of the allergic immune response, most notably as a suppressor of the proinflammatory response by mediating Treg activity.[76–79] Interestingly, the study conducted by Neeland *et al*. (2021) in this cohort found increased proportions of activated Tregs in SA individuals.[62] Further studies investigating immune profiles of MA B-cell subsets and T-cell epigenomes will prove valuable in understanding how B cells and T cells interact in FA.[62]

Strengths and limitations

We recognise the limitations of this study, particularly the relatively small sample size that did not allow for the detection of probes reaching FDR significance. To compensate for this, the analysis approach employed strict cut-offs to obtain the most statistically valid differences between groups; this may have resulted in the omission of subtle differences in DNA methylation or gene expression. We also point out that the MA group in this study was heterogeneous with a range of FAs, with lower rates of asthma (25% of MA individuals were diagnosed with current asthma/wheeze compared with 50% of SA and 56% of NA individuals) and with participants of varying ethnicity (only 38% of MA individuals had both parents born in Australia), which may have resulted in increased variation in the results. However, as the multi-food-allergic population is heterogeneous, a strength of our samples is that they accurately represent the diversity of this community. Additionally, we are aware of the increased number of males in the MA group (75%, while NA and SA groups consisted of 44% and 50% males, respectively). While the linear regression model used for analysis incorporated sex as a covariate, the sex bias in the MA group may still have led to overestimation of sex-specific DMRs in this group (*BRDT* and *SPATA19*), suggesting that these DMRs were detected as a result of sex bias as opposed to allergy status.[80,81] We also note that the data set used to assess enrichment of open chromatin regions in healthy B cells employed samples from adults, which may not reflect the accessible chromatin landscape in adolescents.[27]

CONCLUSION

This study suggests that B cells from peanut-allergic individuals show specific epigenetic and gene expression differences relative to non-allergic individuals. Further, our findings suggest that single-peanut-allergic and MA individuals possess unique group-specific epigenetic signatures that involve differential regulation of B- and T-cell development, B-cell lineage determination and TGF-β signalling pathway, highlighting the phenotypic and molecular distinction of the MA clinical group from single-peanut-allergic individuals. Future research is indicated, particularly around profiling MA patients in order to better characterise this diverse clinical group. It will be interesting to characterise the epigenomes and transcriptomes of other immune cells from matched samples from MA individuals with a range of various FAs, particularly following activation, and integrate these data with assays of functional output.[82]

METHODS

Clinical samples and data collection

This study was carried out using PBMC samples from a subset of adolescents enrolled in the SchoolNuts cohort, a cross-sectional population-based study profiling the prevalence of FA in adolescents between 10 and 14 years of age.[10] Ethics approval to conduct the SchoolNuts study was obtained from the Royal Children's Hospital Research Ethics Committee (no. 31079), the Department of Education and Early Childhood and the Catholic Education Office. Written and informed consent was obtained from the parents of participants in this study. Skin prick tests (SPTs) and oral food challenges (OFCs) were conducted to confirm suspected FA, based on parents' responses to questionnaires regarding history of FA.[10] Participants with a clear and recent history of reaction consistent with IgE-mediated FA, in the context of a positive SPT or sIgE, were not required to undergo an OFC. Blood was collected following clinic visits. Clinic-defined FA was defined as a positive OFC or

convincing recent or severe history in the context of IgE sensitisation (SPT weal size of $\geq$ 3 mm or sIgE $\geq$ 0.35 KuA L^{-1}), as described for the cohort previously. Single-peanut-allergic children had clinic-defined peanut allergy with no evidence of sensitisation or a negative OFC to all other foods. Multi-food-allergic children had clinic-defined peanut allergy with a clinic-defined allergy to at least one other food. Non-food-allergic children had no evidence of sensitisation (< 3 mm) to a panel of 15 food allergens by SPT (egg white, cow's milk, soy, peanut, cashew, almond, hazelnut, walnut, pistachio, macadamia, pecan, Brazil nut, pine nut, sesame and shellfish).

Study participants

The subset used for this study comprised 26 individuals: nine non-food-allergic controls (four males and five females) and 18 food-allergic adolescents (11 males and seven females). In the food-allergic group, 10 (five males and five females) individuals were allergic to only peanut (single-food allergic, SA), while eight (six males and two females) individuals were allergic to more than one food (multi-food allergic, MA) (peanut allergy; along with one or more of the following: tree nuts (either cashew, pistachio, walnut, hazelnut, macadamia, pecan, almond, Brazil nut or pine nut), sesame, egg, milk or shellfish).

Isolation of total B-cell populations and nucleic acid

Peripheral blood mononuclear cells were isolated from blood samples using Ficoll–Paque density gradient centrifugation as previously described, and cryopreserved in liquid nitrogen.[83] PBMCs were viably thawed, and total B-cell populations ($CD3^-CD19^+$) were isolated using fluorescence-activated cell sorting (BD FACS-ARIA Fusion Cell Sorter). Purified B cells were sorted into 350 μL of RLT+2ME (Qiagen) and stored at –80°C for future DNA and RNA extractions. DNA and RNA were extracted from the isolated B cells using the Qiagen AllPrep DNA/RNA Micro Kit, according to the manufacturers' protocols. DNA and RNA were quantified on the Qubit fluorometer using the Qubit dsDNA High Sensitivity (HS) Assay Kit and the Qubit RNA HS Assay Kit (Thermo Fisher Scientific), respectively.

DNA methylation profiling

For DNA methylation profiling, only samples with sufficient DNA yields were used (n = 26). Genomic DNA from 26 samples (200–500 ng) was randomised in a 96-well plate and sent to HuGe-F (Erasmus MC, Rotterdam, the Netherlands) for bisulphite treatment and genome-wide methylation analysis using Illumina Infinium MethylationEPIC BeadChips (the EPIC array). This approach is capable of assessing methylation at over 850 000 CpG sites (EPIC probes) spanning gene bodies, promoters and regulatory elements (ENCODE open chromatin and enhancers).[84]

Raw data were received as.iDAT files, which were preprocessed using the minfi and MissMethyl packages (available from Bioconductor) in the R statistical environment.[85,86] Assessment of sample quality revealed that all samples had a mean detection P-value < 0.01, allowing all samples to remain for analysis. The SWAN (Subset-quantile Within Array Normalization) approach was used to normalise data for technical variation between and within arrays.[87] Probes were then filtered to remove those with poor average quality scores (P-value < 0.01), cross-reactive probes and probes associated with single nucleotide polymorphisms (SNPs).[84] The final data set for analysis comprised methylation data from 770 475 probes. Confounders and covariates were identified using a principal component analysis (Supplementary figure 1) and incorporated into the model for differential analysis. Using available cohort data, covariates with complete data for all individuals were as follows: age, sex, hay fever, wheeze, parent country of birth (within Australia/overseas) and sample position on the EPIC array (plate well and position on chip).

Differential analysis was carried out on the 770 475 probes using the linear regression model with limma.[88] Differentially methylated probes were identified as those showing an unadjusted P-value < 0.05 and a methylation difference ($\Delta\beta$) of > 0.1. The DMRCate tool was used to determine differentially methylated regions (DMRs), and individual probes within these DMRs were identified using Bedtools.[89,90] The nearest genes (within 1Mb of transcription start site in any one direction) to these DMRs and DMPs were determined using the Web-based GREAT tool.[91] Unsupervised clustering analysis of samples was carried out using the k-means clustering function on MeV.[92] DNA methylation data are available on the GEO repository, accession code GSE166067.

Transcriptomic profiling

Isolated RNA was sent for next-generation sequencing on the Illumina NovaSeq 6000 instrument to the Translational Genomics Unit at the Victorian Clinical Genetics Services. Libraries were prepared using the Illumina TruSeq Stranded mRNA Kit with a starting input of 100 ng (where available) and subsequently sequenced, with the generation of approximately 20 million 100-bp paired-end reads per sample. Raw data were received as fastq files, which were subject to a quality check using FastQC, with all samples passing QC. Reads were then aligned to the human transcriptome (GrCh37 v70) using Bowtie, and gene expression estimates were derived using the mmseq pipeline.[93,94] Genes exhibiting low expression (mean reads per kilobase of transcript, per million of mapped reads (RPKM) < 1 across all samples) were removed from the analysis, leaving expression data for 20 025 genes. The DESeq package (available on Bioconductor) was used to normalise count data and determine gene expression differences between group comparisons.[95] RNA-seq data are available on the GEO repository, accession code GSE165316.

Regulatory element analysis

DMPs found in NA vs SA vs MA comparisons (Figure 3a), and DMPs overlapping open chromatin regions in B cells

(Supplementary figure 4) were scanned for transcription factor-binding motifs using HOMER.[96] As a result of differing functionalities of proximal and distal regulatory elements, DMPs were classified as those that mapped to proximal regions (within 5 Kb of a gene transcription start site (TSS)) and those more distal (beyond 5 Kb of TSS) (Figure 6a). DMPs were further grouped according to whether higher or lower methylation was observed in FA groups.

A 200-bp region around the distal DMPs was scanned for transcription factor-binding motifs using HOMER (findMotifsGenome.pl). Motifs were identified as those showing enrichment of 10% over random background in at least one of the groups, and a fold change (FC) of 1.5 compared with background sequences. Proximal DMPs were scanned for enriched TF motifs using HOMER (findMotifs.pl). However, given the relatively low numbers of proximal DMPs and therefore associated genes, enrichment cut-offs were set to more stringent standards to minimise false positives. In this case, enrichment was defined as motifs showing $\geq$ 15% enrichment and an FC $\geq$ 2, over background, with a *P*-value $<$ 0.05.

DEGs found in NA vs SA vs MA comparisons were similarly scanned for enriched TFs using HOMER (findMotifs.pl), with enrichment defined as motifs showing $\geq$ 15% enrichment and an FC $\geq$ 2 over background, with a *P*-value $<$ 0.1 (Figure 6c).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Samira Imran: Formal analysis; Methodology; Writing-original draft. **Melanie Neeland:** Conceptualization; Funding acquisition; Methodology; Resources; Supervision; Writing-review & editing. **Jennifer Koplin:** Data curation; Project administration; Writing-review & editing. **Shyamali Dharmage:** Project administration; Resources; Writing-review & editing. **Mimi LK Tang:** Project administration; Resources; Writing-review & editing. **Susan Sawyer:** Project administration; Resources; Writing-review & editing. **Thanh Dang:** Methodology; Project administration; Resources; Writing-review & editing. **Vicki McWilliam:** Data curation; Resources; Writing-review & editing. **Rachel Peters:** Data curation; Project administration; Resources; Writing-review & editing. **Kirsten P Perrett:** Data curation; Project administration; Supervision; Writing-review & editing. **Boris Novakovic:** Conceptualization; Funding acquisition; Methodology; Supervision; Writing-review & editing. **Richard Saffery:** Conceptualization; Funding acquisition; Methodology; Resources; Supervision; Writing-review & editing.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.