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# Differential epigenome-wide DNA methylation patterns in childhood obesity-associated asthma

## SUBJECT AREAS:

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RESEARCH

RESPIRATORY TRACT DISEASES

OBESITY

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While DNA methylation plays a role in T-helper (Th) cell maturation, its potential dysregulation in the non-atopic Th1-polarized systemic inflammation observed in obesity-associated asthma is unknown. We studied DNA methylation epigenome-wide in peripheral blood mononuclear cells (PBMCs) from 8 obese asthmatic pre-adolescent children and compared it to methylation in PBMCs from 8 children with asthma alone, obesity alone and healthy controls. Differentially methylated loci implicated certain biologically relevant molecules and pathways. PBMCs from obese asthmatic children had distinctive DNA methylation patterns, with decreased promoter methylation of *CCL5*, *IL2RA* and *TBX21*, genes encoding proteins linked with Th1 polarization, and increased promoter methylation of *FCER2*, a low-affinity receptor for IgE, and of *TGFB1*, inhibitor of Th cell activation. T-cell signaling and macrophage activation were the two primary pathways that were selectively hypomethylated in obese asthmatics. These findings suggest that dysregulated DNA methylation is associated with non-atopic inflammation observed in pediatric obesity-associated asthma.

Obesity and asthma are common and debilitating conditions that are especially prevalent in urban Hispanic and African American populations<sup>1,2</sup>. Over the past decade, obesity is recognized as an independent risk factor for asthma<sup>3–7</sup> and is associated with greater morbidity compared to asthma in normal-weight children<sup>8,9</sup>. Mechanistic studies suggest that obesity-associated asthma is a distinct entity, but its pathophysiology remains poorly defined<sup>10</sup>.

While obesity is a chronic inflammatory state characterized by leptin-mediated T helper (Th)1 inflammation<sup>11</sup>, “classic” childhood asthma is predominantly allergic in nature with a relative bias toward a Th2 phenotype<sup>12</sup>. In contrast to Th2 inflammation, non-atopic inflammation<sup>13,14</sup> with Th1 polarization<sup>15</sup> which correlated with lower airway obstruction has been reported in obese asthmatics<sup>15</sup>, lending support to the hypothesis that obesity-associated asthma is a distinct asthma phenotype.

Both asthma and obesity are multifactorial conditions and likely reflect a combined effect of genetic and environmental modulation<sup>16,17</sup>. Epigenetic dysregulation manifested by DNA methylation, potentially reflecting the combined genetic and environmental modulation, has been reported in context of both obesity and asthma alone<sup>16,17</sup>. DNA methylation also plays a role in Th cell differentiation<sup>18</sup>. However, the potential dysregulation of DNA methylation associated with non-atopic Th-1 polarized systemic inflammation in obesity-associated asthma have not been investigated. To address this gap in knowledge, in a pilot study, we performed an epigenome-wide association study to investigate DNA methylation in Peripheral blood mononuclear cells (PBMCs) obtained from obese asthmatic children, comparing it to methylation in PBMCs from children with asthma alone, obesity alone and healthy control individuals. We hypothesized that obese asthmatic PBMCs have distinct epigenome-wide DNA methylation patterns that are associated with Th cell differentiation when compared to the other three study groups. We also proposed to study whether inflammatory pathways activated in the context of obesity-associated asthma are distinct from those activated in normal weight children with asthma and those with obesity alone. Although designed as a pilot study, our results are strongly suggestive of a role for the epigenome in mediating risk associated with obesity in the causation of asthma.

## Results

**Study population.** Demographic characteristics did not significantly differ between the four study groups (Table 1), other than mean weight and BMI z-score, in keeping with the study design. Asthma severity also



Table 1 | Characteristics of the study population

Variable*	Obese asthmatics	Normal-weight asthmatics	Obese non-asthmatics	Healthy controls	P value
Age (years)	9.1±1.4	8.1±1.2	9.3±1.3	8.3±1.4	0.07
Weight (kgs)	51.4±15.7	32.3±7.4	48.2±10.8	33.2±6.3	<0.01
BMI z score	2±0.5	0.6±1.2	1.7±0.4	0.7±0.5	<0.01
Gender (M:F)	3:5	3:5	4:4	3:5	0.6
Ethnicity (H:AA)	3:5	3:5	4:4	3:5	0.6
Percent predicted FEV <sub>1</sub> /FVC ratio	82.6±5.4	87.9±5.1	89.4±3.6	89.9±6.7	0.048

BMI body mass index, M male, F female, H Hispanic, AA African-American.

Age, weight, BMI z-score and percent predicted FEV<sub>1</sub>/FVC ratio are reported as mean±SD.

did not differ between the obese asthmatics and normal-weight asthmatics ( $p = 0.7$ ). Among obese asthmatics, 12.5% had intermittent, 37.5% had mild persistent, 25% had moderate persistent and 25% had severe persistent asthma while among normal-weight asthmatics, 25% had intermittent, 25% had mild persistent, 25% had moderate persistent and 25% had severe persistent asthma.

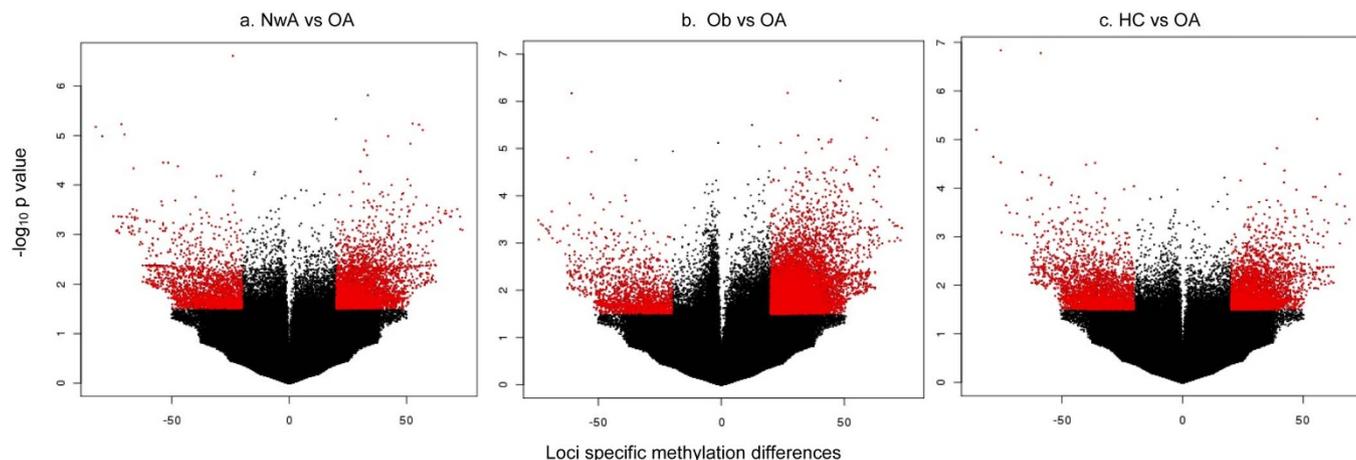
**Epigenome-wide DNA methylation patterns in obese asthmatics compared to normal weight asthmatics, obese non-asthmatics and healthy controls.** DNA methylation profile in PBMCs from obese asthmatics was distinct from the profile in PBMCs from normal-weight asthmatics, obese non-asthmatics and healthy controls as seen on volcano plots (Figure 1) and heat maps (Figure 2a). While 7119 loci were differentially methylated in PBMCs from obese asthmatics compared to those from normal-weight asthmatics, 12,875 were differentially methylated compared to PBMCs from obese non-asthmatics and 6773 were differentially methylated when compared to healthy controls (Figure 2b). Two hundred and fifteen loci were consistently differentially methylated in obese asthmatic PBMCs compared to the other three study groups. The top fifty differentially methylated promoter loci with an angle difference of greater than 20 (~20% difference in methylation<sup>19</sup>) in PBMCs from obese asthmatics compared to those from normal-weight asthmatics, obese non-asthmatics and healthy controls are summarized in Table 2,3,4.

Cell to cell signaling and T lymphocyte differentiation were the primary functions of the genes targeted at their promoters for differential methylation that were identified by IPA analysis in PBMCs from obese asthmatics compared to normal-weight asthmatics and healthy controls (Table 5). Compared with normal-weight asthmatics and healthy controls, PBMCs from obese asthmatics had

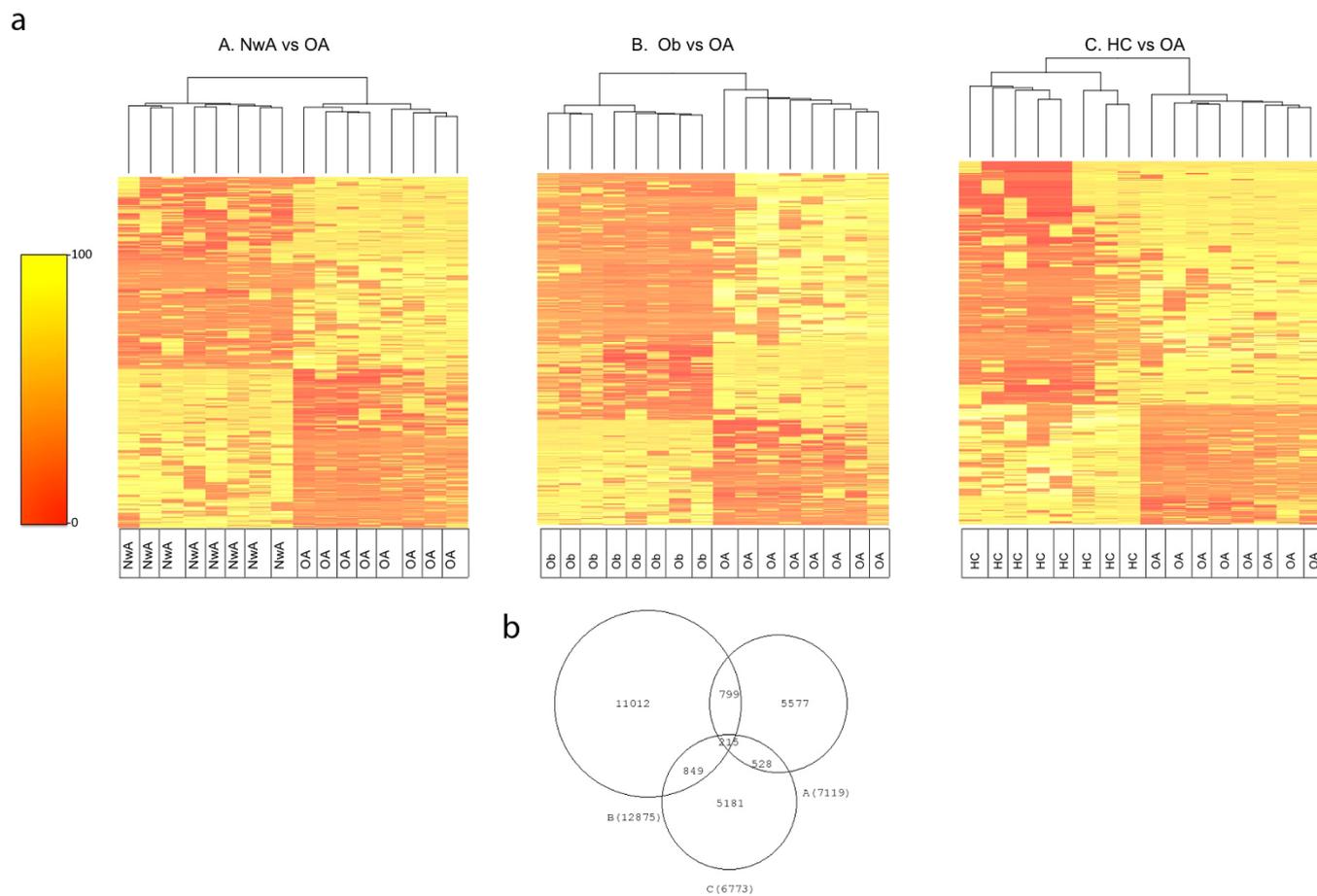
decreased methylation of gene promoters associated with Th cell differentiation (*ADAM17*, *LIF*, *NHEJ1*, *AHR*, *IL2RA*, *TBX21*, and *CCL5* (*RANTES*)). Conversely, gene promoters of *FCER2* (*CD23*), a low affinity receptor for IgE and *TGFBI*, encoding for TGF $\beta$ , secreted by T regulatory cells (Tregs) that controls Th cell differentiation, were hypermethylated in PBMCs from obese asthmatics, compared to normal-weight asthmatics and healthy controls respectively, in keeping with the observed higher Th cell mediated inflammation<sup>15,20</sup>. Compared to obese non-asthmatics, genes encoding *CCL5*, *PGDR* and *PI3K*, known to influence chemotaxis of natural killer cells and macrophages were hypomethylated and *GNA12* and *z*, members of the G protein family that are ubiquitous in the intracellular signaling pathways were hypermethylated in PBMCs from obese asthmatics.

#### Differentially methylated pathways in obesity associated asthma.

To obtain information about the functional relevance of DNA methylation differences observed at the molecular level, we applied IPA to identify the canonical pathways selected for DNA methylation targeting in PBMCs from obese asthmatics compared to normal-weight asthmatics, obese non-asthmatics and healthy controls. In keeping with the gene functions identified to be modulated by DNA methylation, the primary canonical pathways found to be differentially methylated in PBMCs from obese asthmatics compared to normal-weight asthmatics including T cell signaling, and CCR5 signaling and nitric oxide and reactive oxygen release from macrophages (Figure 3a). As compared to both PBMCs from obese non-asthmatics and healthy controls (Fig. 3b and 3c), obese asthmatics had differential activation of pathways associated with 4-IBB signaling and regulation of activation of IL-2 expression on T lymphocytes. In addition to those discussed above, we retained other pathways in the figure as these pathways appear to be of



**Figure 1 | Volcano plots comparing DNA methylation in obese asthmatics (OA) to (a). Normal-weight Asthmatics (NwA) (b). Obese non-asthmatics (Ob) and (c). Healthy Controls (HC) (plot of loci specific methylation differences (x-axis) vs.  $-\log_{10}$  p value (y-axis)). The red dots represent the top 1% loci where the methylation difference between the two study groups was greater than 20.**



**Figure 2** | (a). Heat maps of methylation differences discriminating obese asthmatics (OA) from (A) normal weight asthmatics (NwA), (B) obese non-asthmatics (Ob) and (C) healthy controls (HC). Red indicates more methylation and yellow less methylation by HELP-tagging. The columns represent subjects in each study group while the rows are the loci where the methylation difference between the two study groups being compared was greater than 20. (b). Proportional Venn diagram depicting overlap of differentially methylated CpG sites identified when obese asthmatics (OA) were compared to (A) normal-weight asthmatics (NwA), (B) Obese non-asthmatics (Ob) and (C) healthy controls (HC). Two hundred and fifteen loci were consistently differentially methylated in obese asthmatics compared to the other three groups.

physiologic significance and it would be interesting if the same pathways were enriched in a larger sample size.

In addition to canonical pathways, we also mapped the molecular interaction networks utilizing IPA software. In the network comparing obese asthmatics to normal-weight asthmatics, we found that while the  $IFN\gamma$  promoter itself was not differentially methylated, it was the hub of the enriched pathways and was associated with other differentially methylated factors (Figure 4a). Further, while factors associated with known obesity-specific innate immune mechanisms (including *ALOX15*, *IGFB4*, *SOCS2* and *SOCS3*) were hypomethylated in obese non-asthmatics, obese asthmatics had evidence of hypomethylation of *IFNG*, *CCL5* and *PPARG* promoters, cytokines and transcription factors associated with both innate and non-atopic adaptive immunity (Figure 4b). Similarly, as compared to healthy controls, promoters of *CCL5* and *CSF1* gene, associated with macrophage mediated inflammation and differentiation and survival respectively, was hypomethylated in PBMCs from obese asthmatics (Figure 4c).

## Discussion

In this pilot study, we found evidence to support the hypothesis that PBMCs derived from pre-adolescent obese asthmatic minority children have distinct patterns of DNA methylation differing from those observed in PBMCs from normal weight asthmatics, obese non-asthmatics and healthy controls. There was decreased promoter methylation of a subset of genes that encode for molecules associated with

innate immune and non-atopic patterns of inflammation, including *CCL5*<sup>21</sup>, *IL2RA*<sup>22</sup>, and *TBX21*<sup>23</sup>, a transcription factor associated with increased Th1 differentiation, while promoter methylation of *FCER* (CD23), a low affinity receptor for IgE<sup>24</sup> and of *TGFB1*, released by Tregs to control T cell mediated inflammation<sup>25</sup>, was increased. Together, differential methylated molecules in PBMCs from obese asthmatic children were associated with pathways linked to T cell differentiation and increased macrophage activation, the two primary cell types that have been linked to the pathophysiology of obesity-associated asthma<sup>20,26</sup>. We have previously demonstrated that obesity-associated asthma is associated with Th1 polarization in this study sample, with increased Th1/Th2 cell ratio when compared to normal-weight asthmatics<sup>15</sup>, providing a biological pathway for prior reports of non-atopic pattern of inflammation in obesity-associated asthma<sup>13,27</sup>. Epigenome wide methylation patterns found in the current study suggest that DNA methylation may play a role in the observed Th1 polarization among obese asthmatics.

We utilized an epigenome-wide methylation assay that yielded quantitative DNA methylation data on ~2.0 million loci. Previous studies performed in our laboratory using this technology have reported a strong correlation between genome-wide and single locus methylation<sup>19,28,29</sup>, supporting the inferences drawn from the epigenome-wide studies. Our approach is different from those previously utilized in epigenetic studies done in context of asthma where single gene promoter methylation sites were studied among asthmatics using bisulphite MassArray or Pyrosequencing<sup>30</sup>. In addition to



Table 2 | Top fifty promoter loci with differential methylation between obese asthmatics and normal weight asthmatics

ID	Symbol	Entrez Gene Name	Methylation HpaII angle difference
Hypermethylated loci			
NM_032808	LINGO1	leucine rich repeat and Ig domain containing 1	-26.75
NM_002457	MUC2	mucin 2, oligomeric mucus/gel-forming	-30.20
NM_175875	SIX5	SIX homeobox 5	-30.22
NM_000674	ADORA1	adenosine A1 receptor	-30.82
NM_012212	PTGR1	prostaglandin reductase 1	-36.04
NM_004292	RIN1	Ras and Rab interactor 1	-36.58
NM_030923	TMEM163	transmembrane protein 163	-36.66
NR_023315	ASZ1	ankyrin repeat, SAM and basic leucine zipper domain containing 1	-37.41
NM_016396	CTDSPL2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A)	-39.70
NM_000577	IL1RN	interleukin 1 receptor antagonist	-41.41
NM_005514	HLA-B	major histocompatibility complex, class I, B	-46.27
NM_004979	KCND1	potassium voltage-gated channel, Shal-related subfamily, member 1	-46.61
NM_002002	FCER2	Fc fragment of IgE, low affinity II, receptor for (CD23)	-47.49
NM_003327	TNFRSF4	tumor necrosis factor receptor superfamily, member 4	-46.31
NM_002617	PEX10	peroxisomal biogenesis factor 10	-47.88
NM_014700	RAB11FIP3	RAB11 family interacting protein 3 (class II)	-48.80
NM_139172	TMEM190	transmembrane protein 190	-50.02
NM_006667	PGRMC1	progesterone receptor membrane component 1	-51.12
NM_032790	ORAI1	ORAI calcium release-activated calcium modulator 1	-51.61
NM_002384	MBD1	methyl-CpG binding domain protein 1	-53.69
NM_016396	CTDSPL2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A)	-54.03
NM_020343	RALGAPA2	Ral GTPase activating protein, alpha subunit 2 (catalytic)	-54.91
NM_022143	LRRC4	leucine rich repeat containing 4	-58.71
NM_024617	ZCCHC6	zinc finger, CCHC domain containing 6	-59
NM_001769	CD9	CD9 molecule	-63.28
Hypomethylated loci			
NM_004356	CD81	CD81 molecule	22.89
NM_005905	SMAD9	SMAD family member 9	24.21
NM_002985	CCL5	chemokine (C-C motif) ligand 5	25.65
NM_006202	PDE4A	phosphodiesterase 4A, cAMP-specific	25.78
NM_198836	ACACA	acetyl-CoA carboxylase alpha	30.10
NM_006253	PRKAB1	protein kinase, AMP-activated, beta 1 non-catalytic subunit	30.29
NM_000229	LCAT	lecithin-cholesterol acyltransferase	30.45
NM_181524	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	32.71
NM_024782	NHEJ1	nonhomologous end-joining factor 1	35.34
NM_000283	PDE6B	phosphodiesterase 6B, cGMP-specific, rod, beta	35.76
NM_017617	NOTCH1	notch 1	38.86
NM_007161	LST1	leukocyte specific transcript 1	42.42
NM_016592	GNAS	GNAS complex locus	42.82
NM_006601	PTGES3	prostaglandin E synthase 3 (cytosolic)	42.85
NM_002641	PIGA	phosphatidylinositol glycan anchor biosynthesis, class A	47.80
NM_003540	HIST1H4A	histone cluster 1, H4a	47.36
NM_001807	CEL	carboxyl ester lipase (bile salt-stimulated lipase)	47.79
NM_016541	GNG13	guanine nucleotide binding protein (G protein), gamma 13	47.74
NM_030928	CDT1	chromatin licensing and DNA replication factor 1	48.15
NM_213604	ADAMTSL5	ADAMTS-like 5	50.41
NM_022143	LRRC4	leucine rich repeat containing 4	52.43
NM_006668	CYP46A1	cytochrome P450, family 46, subfamily A, polypeptide 1	56.03
NM_002079	GOT1	glutamic-oxaloacetic transaminase 1,	56.28
NM_013361	ZNF223	zinc finger protein 223	61
NM_003073	SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin	64.52

obtaining promoter-specific methylation data by our approach, we were able to obtain methylation profiles on other genes not routinely studied in the context of obesity or asthma. These methylation profiles were used to elucidate the interaction between the products of the differentially methylated genes, thereby identifying pathways that are modulated by DNA methylation and play a role in the inflammatory patterns observed among obese asthmatics. This information provides a more comprehensive insight into the complex interactions between inflammatory pathways activated by the co-existence of two chronic inflammatory conditions, asthma and obesity.

In keeping with the atopic phenotype of asthma among normal weight individuals, prior studies of DNA methylation profiles performed in asthmatics have found decreased methylation of IL-4 promoter and increased methylation of IFN $\gamma$  promoter in adults<sup>30</sup> and children<sup>31</sup>, confirming the role of methylation in naive Th cell maturation<sup>32</sup> and in asthma. Differential methylation has correlated with cytokine production by the cells providing evidence of the functional implications of methylation<sup>30,31</sup>. Recently, using massively-parallel sequencing, Kim et al. have demonstrated differential methylation in bronchial mucosa from atopic adults with asthma compared to non-atopic asthmatics, providing additional support for the role of



Table 3 | Top fifty promoter loci with differential methylation between obese asthmatics and obese non-asthmatics

ID	Symbol	Entrez Gene Name	Methylation HpaII angle difference
Hypermethylated loci			
NM_000101	CYBA	cytochrome b-245, alpha polypeptide	-21.66
NM_006961	ZNF19	zinc finger protein 19	-22.11
NM_015470	RAB11FIP5	RAB11 family interacting protein 5 (class I)	-22.53
NM_000755	CRAT	carnitine O-acetyltransferase	-26.18
NM_004064	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	-28.04
NM_080649	APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	-28.60
NM_004791	ITGBL1	integrin, beta-like 1 (with EGF-like repeat domains)	-29.08
NM_006743	RBM3	RNA binding motif (RNP1, RRM) protein 3	-29.72
NM_005054	RGPD5	RANBP2-like and GRIP domain containing 5	-31.06
NM_002208	ITGAE	integrin, alpha E (antigen CD103, human mucosal lymphocyte antigen)	-33.12
NM_002002	FCER2	Fc fragment of IgE, low affinity II, receptor for (CD23)	-35.03
NM_019625	ABCB9	ATP-binding cassette, sub-family B (MDR/TAP), member 9	-36.66
NM_152740	HIBADH	3-hydroxyisobutyrate dehydrogenase	-39.92
NM_006332	IFI30	interferon, gamma-inducible protein 30	-43.98
NM_183240	TMEM37	transmembrane protein 37	-44.07
NM_001805	CEBPE	CCAAT/enhancer binding protein (C/EBP), epsilon	-44.37
NM_005037	PPARG	peroxisome proliferator-activated receptor gamma	-44.94
NM_002774	KLK6	kallikrein-related peptidase 6	-45.46
NM_003486	SLC7A5	solute carrier family 7 (amino acid transporter light chain, L system),	-47.79
NM_033023	PDGFA	platelet-derived growth factor alpha polypeptide	-48.40
NM_024552	CERS4	ceramide synthase 4	-48.55
NM_004104	FASN	fatty acid synthase	-49.08
NM_006793	PRDX3	peroxiredoxin 3	-51.71
NM_172364	CACNA2D4	calcium channel, voltage-dependent, alpha 2/delta subunit 4	-57.17
NM_004472	FOXD1	forkhead box D1	-61.60
Hypomethylated loci			
NM_003513	HIST1H2AB/HIST1H2AE	histone cluster 1, H2ae	24.78
NM_080816	SIRPG	signal-regulatory protein gamma	26.70
NM_001374	DNASE1L2	deoxyribonuclease I-like 2	26.96
NR_023391	CYP4F30P	cytochrome P450, family 4, subfamily F, polypeptide 30, pseudogene	29.46
NM_005003	NDUFAB1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa	30.03
NM_005265	GGT1	gamma-glutamyltransferase 1	30.76
NM_002718	PPP2R3A	protein phosphatase 2, regulatory subunit B', alpha	31.16
NM_020686	ABAT	4-aminobutyrate aminotransferase	31.99
NM_002066	GML	glycosylphosphatidylinositol anchored molecule like protein	35.78
NM_001444	FABP5	fatty acid binding protein 5 (psoriasis-associated)	35.79
NM_181524	PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	36.31
NM_002745	MAPK1	mitogen-activated protein kinase 1	36.33
NM_005855	RAMP1	receptor (G protein-coupled) activity modifying protein 1	39.95
NM_012461	TINF2	TERF1 (TRF1)-interacting nuclear factor 2	42.13
NM_005037	PPARG	peroxisome proliferator-activated receptor gamma	44.94
NM_000498	CYP11B2	cytochrome P450, family 11, subfamily B, polypeptide 2	45
NM_178011	LRRTM3	leucine rich repeat transmembrane neuronal 3	45.43
NM_005063	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	47.58
NM_007161	LST1	leukocyte specific transcript 1	48.37
NM_004566	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	52.96
NR_026790	HCG11	HLA complex group 11	53.02
NM_013361	ZNF223	zinc finger protein 223	55.25
NM_173821	CXXC11	CXXC finger protein 11	55.43
NM_006099	PIAS3	protein inhibitor of activated STAT, 3	55.46
NM_017693	BIVM	basic, immunoglobulin-like variable motif containing	56.67
NM_015320	ARHGEF4	Rho guanine nucleotide exchange factor (GEF) 4	71.73

DNA methylation in asthma pathogenesis<sup>33</sup>. Consistent with our findings of Th1 polarization with lower serum levels of IL-13 and higher levels of IL-6 and TNF $\alpha$ , as well as higher Th1/Th2 cell ratio in obese asthmatic pre-adolescent children<sup>15</sup>, we found hypomethylation of *CCL5*, a pro Th1 chemokine<sup>21</sup> that has strong chemotaxis not only for naive and memory T cells but also for macrophages, the primary cell associated with obesity mediated inflammation<sup>34</sup>. Similarly, *TBX21*, a transcription factor involved in selective differentiation of naive Th cells into Th1 cells<sup>23</sup> was also hypomethylated in PBMCs from obese asthmatics relative to healthy controls.

However, since similar differences in *TBX21* methylation were not observed between obese and normal-weight asthmatics, it is possible that differential methylation of other pathways, such as those mediated by GATA3, a Th2 transcription factor or downstream molecules such as IL-4, IL-5 or IL-13, may also play a role in the observed Th1 polarization among obese asthmatics relative to normal-weight asthmatics. Conversely, there was evidence of hypermethylation of IgE receptor CD23<sup>24</sup> in obese asthmatics compared to normal-weight asthmatics. CD23 is proposed to play a role in antigen presentation and regulation of immune responses, particularly with enhancement



Table 4 | Top fifty promoter loci with differential methylation between obese asthmatics and healthy controls

ID	Symbol	Entrez Gene Name	Methylation HpaII angle difference
<b>Hypermethylated loci</b>			
NM_005004	NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex	-21.89
NM_016538	SIRT7	sirtuin 7	-27.80
NM_017675	CDHR2	cadherin-related family member 2	-35.42
NM_178496	MB21D2	Mab-21 domain containing 2	-36.33
NM_002896	RBM4	RNA binding motif protein 4	-38.57
NM_000422	KRT17	keratin 17	-43.98
NM_002774	KLK6	kallikrein-related peptidase 6	-44.02
NM_012338	TSPAN12	tetraspanin 12	-44.49
NM_181553	CMTM3	CKLF-like MARVEL transmembrane domain containing 3	-44.46
NM_052885	SLC2A13	solute carrier family 2 (facilitated glucose transporter), member 13	-47.89
NM_182527	CABP7	calcium binding protein 7	-48.07
NM_024641	MANEA	mannosidase, endo-alpha	-48.67
NM_006907	PYCR1	pyrroline-5-carboxylate reductase 1	-49.26
NM_052910	SLITRK1	SLIT and NTRK-like family, member 1	-52.37
NM_198570	VWC2	von Willebrand factor C domain containing 2	-52.42
NM_138973	BACE1	beta-site APP-cleaving enzyme 1	-55.56
NM_014578	RHOD	ras homolog family member D	-55.69
NM_001329	CTBP2	C-terminal binding protein 2	-56.93
NR_027052	THAP7-AS1	THAP7 antisense RNA 1 (non-protein coding)	-57.29
NM_001332	CTNND2	catenin (cadherin-associated protein),	-57.40
NM_006234	POLR2J	polymerase (RNA) II (DNA directed) polypeptide J	-57.44
NM_001395	DUSP9	dual specificity phosphatase 9	-57.75
NM_005938	FOXO4	forkhead box O4	-58.65
NM_001880	ATF2	activating transcription factor 2	-62.41
NM_020781	ZNF398	zinc finger protein 398	-68.76
<b>Hypomethylated loci</b>			
NM_001571	IRF3	interferon regulatory factor 3	20.49
NM_172241	CTAGE1	cutaneous T-cell lymphoma-associated antigen 1	20.50
NM_006290	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	21.22
NM_014898	ZFP30	zinc finger protein 30 homolog	22.13
NM_000131	F7	coagulation factor VII	22.74
NM_003463	PTP4A1	protein tyrosine phosphatase type IVA, member 1	23.28
NM_024693	ECHDC3	enoyl CoA hydratase domain containing 3	25.56
NR_026918	PTPN3	protein tyrosine phosphatase, non-receptor type 3	26.80
NM_005905	SMAD9	SMAD family member 9	27.19
NR_001297	PCDHGB8P	protocadherin gamma subfamily B, 8 pseudogene	27.35
NM_031849	PCDHA6	protocadherin alpha 6	28.97
NM_014632	MICAL2	microtubule associated monooxygenase,	32.76
NM_014330	PPP1R15A	protein phosphatase 1, regulatory subunit 15A	34.37
NR_003716	HOTAIR	HOX transcript antisense RNA	35.79
NM_178012	TUBB2B	tubulin, beta 2B class IIb	37.26
NM_178172	GPIHBP1	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	38.18
NM_015257	TMEM194A	transmembrane protein 194A	38.31
NM_001276	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	41.25
NM_000853	GSTT1	glutathione S-transferase theta 1	41.85
NM_145726	TRAF3	TNF receptor-associated factor 3	51.74
NR_021485	EGFEM1P	EGF-like and EMI domain containing 1	55.82
NM_020713	ZNF512B	zinc finger protein 512B	58.40
NM_013361	ZNF223	zinc finger protein 223	58.42
NM_024014	HOXA6	homeobox A6	59.92
NM_052945	TNFRSF13C	tumor necrosis factor receptor superfamily, member 13C	64.29

of IgE mediated T and B cell responses<sup>24</sup>. Together these results provide evidence that observed patterns of DNA methylation likely play a role in non-atopic inflammatory phenotype of obesity-associated asthma.

Stefanowicz et al.<sup>35</sup> recently demonstrated cell-specific methylation differences in context of asthma and atopy, a systemic disease, highlighting the need for simultaneous investigation of DNA methylation in cells obtained from systemic circulation and from disease-targeted organs. They, however, did not find any significant methylation differences in PBMCs obtained from asthmatics and healthy controls, findings that differ from our observations. There may be

several factors contributing to the observed differences. There were inherent differences in the technique used to elucidate methylation patterns. While our method using DNA methylation specific restriction enzymes followed by ligation mediated PCR amplification allows the study of ~2 million loci, Stefanowicz et al. investigated methylation at 1505 loci on bisulphite treated DNA using a cancer panel, potentially limiting their ability to identify differential methylation at several additional loci. Moreover, our study cohort included older children, so inherent differences in age, as well as potential differences in ethnicity and asthma severity between the two study samples may offer additional explanations for the differences. Prior



**Table 5 | Molecules with differentially methylated gene promoters and their associated cellular functions in obese asthmatics (OA) compared to a) normal weight asthmatics (NwA), b) obese non-asthmatics (Ob) and c) healthy controls (HC)**

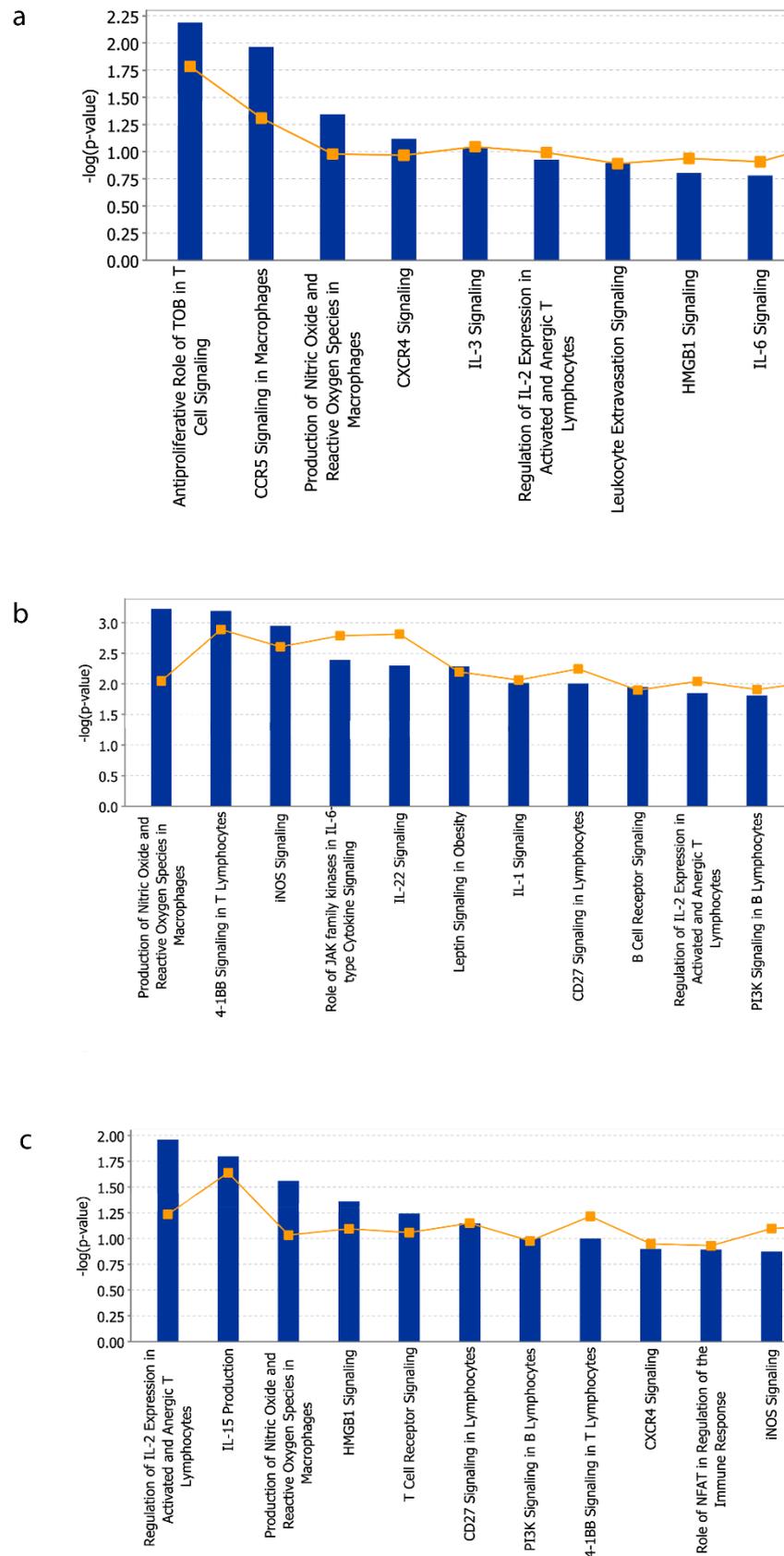
OA vs NwA	OA vs Ob	OA vs HC
Function: Cell to cell signaling	Function: Chemotaxis of NK cells	Function: Differentiation of T lymphocytes
Hypomethylated gene promoters	Hypomethylated gene promoters	Hypomethylated gene promoters
a. Adam metalloproteinase domain ( <i>ADAM17</i> ) (pro-inflammatory enzyme causing ectodomain shedding of IL6 and TNF $\alpha$ ) <sup>46</sup>	a. <i>CCL5</i>	a. <i>ADAM17</i>
b. Interleukin 2 receptor alpha ( <i>IL2RA</i> ) (member of high-affinity receptor for IL2, associated with increased Th1 differentiation) <sup>22</sup>	b. Prostaglandin D2 receptor ( <i>PGDR</i> ) (Regulates prostaglandin D mediated mast cell and Th cell responses) <sup>53</sup>	b. <i>IL2RA</i>
c. Interleukin 27 ( <i>IL27</i> ) (induces maturation of T regulatory cells) <sup>47</sup>	c. G protein alpha 11 ( <i>GNA11</i> ) (involved in receptor mediated activation of signaling pathways) <sup>54</sup>	c. <i>AHR</i>
d. Leukemia inhibitory factor ( <i>LIF</i> ) (Proliferation of mature T cells) <sup>48</sup>	d. Phosphatidol inositol 3 kinase ( <i>PI3K</i> ) (involved in activation of signaling pathways) <sup>39</sup>	d. T box transcription factor ( <i>TBX21</i> ) (Th1 lineage defining transcription factor) <sup>23</sup>
e. Non homologous end joining factor 1 ( <i>NHEJ1</i> ) (associated with DNA repair; deficiency linked to immunodeficiency) <sup>49</sup>	Hypermethylated gene promoters	e. PRELI domain 1 (expressed in peripheral B lymphocytes and regulates STAT6) <sup>56</sup>
f. chemokine (C-C motif) ligand 5 ( <i>CCL5</i> ) (chemoattractant for mast cells, memory T cells) <sup>21</sup>	e. G protein alpha 12 ( <i>GNA12</i> ) (involved in receptor mediated activation of signaling pathways) <sup>54</sup>	f. <i>CCL5</i>
Hypermethylated gene promoters	f. G protein alpha z polypeptide ( <i>GNAz</i> ) (involved in receptor mediated activation of signaling pathways) <sup>54</sup>	g. Colony stimulating factor 1 receptor ( <i>CSF1R</i> ) (macrophage production and differentiation) <sup>57</sup>
g. Aryl hydrocarbon receptor ( <i>AHR</i> ) (regulates immune system, and cellular proliferation and migration) <sup>50</sup>	g. TNF receptor superfamily 1B ( <i>TNFSRF1B</i> ) (increased T cell and macrophage related inflammation) <sup>52</sup>	g. Colony stimulating factor 1 receptor ( <i>CSF1R</i> ) (macrophage production and differentiation) <sup>57</sup>
h. Chromodomain helicase DNA binding protein ( <i>CHD</i> ) <sup>51</sup>	h. IFN $\gamma$ receptor 1 ( <i>IFNGR1</i> ) (binding site for IFN $\gamma$ , site for interaction between monocytes and Tcells) <sup>55</sup>	Hypermethylated gene promoters
i. TNF receptor superfamily 4 ( <i>TNFSRF4</i> ) (regulate development of memory and effector Tcells) <sup>52</sup>	i. Fc $\epsilon$ RII ( <i>FCER</i> ), low affinity receptor for IgE <sup>24</sup>	h. <i>CHD</i>
j. TNF receptor superfamily 9 ( <i>TNFSRF9</i> ) (activation of mast cells, CD4+ and CD8+ cells) <sup>52</sup>		i. TGF beta ( <i>TGFB1</i> ) (produced by T regulatory cells to regulate immune responses) <sup>25</sup>
k. Fc $\epsilon$ RII ( <i>FCER</i> ), low affinity receptor for IgE <sup>24</sup>		j. <i>TNFSRF4</i>

studies investigating the influence of ethnicity on clinical phenotypes have identified ethnicity-specific single nucleotide polymorphisms that correlate with clinical parameters including peripheral white cell counts<sup>36</sup> and disease such as multiple sclerosis<sup>37</sup>. Whether similar ethnicity-specific differences in the epigenome are associated with disease incidence and severity needs further investigation.

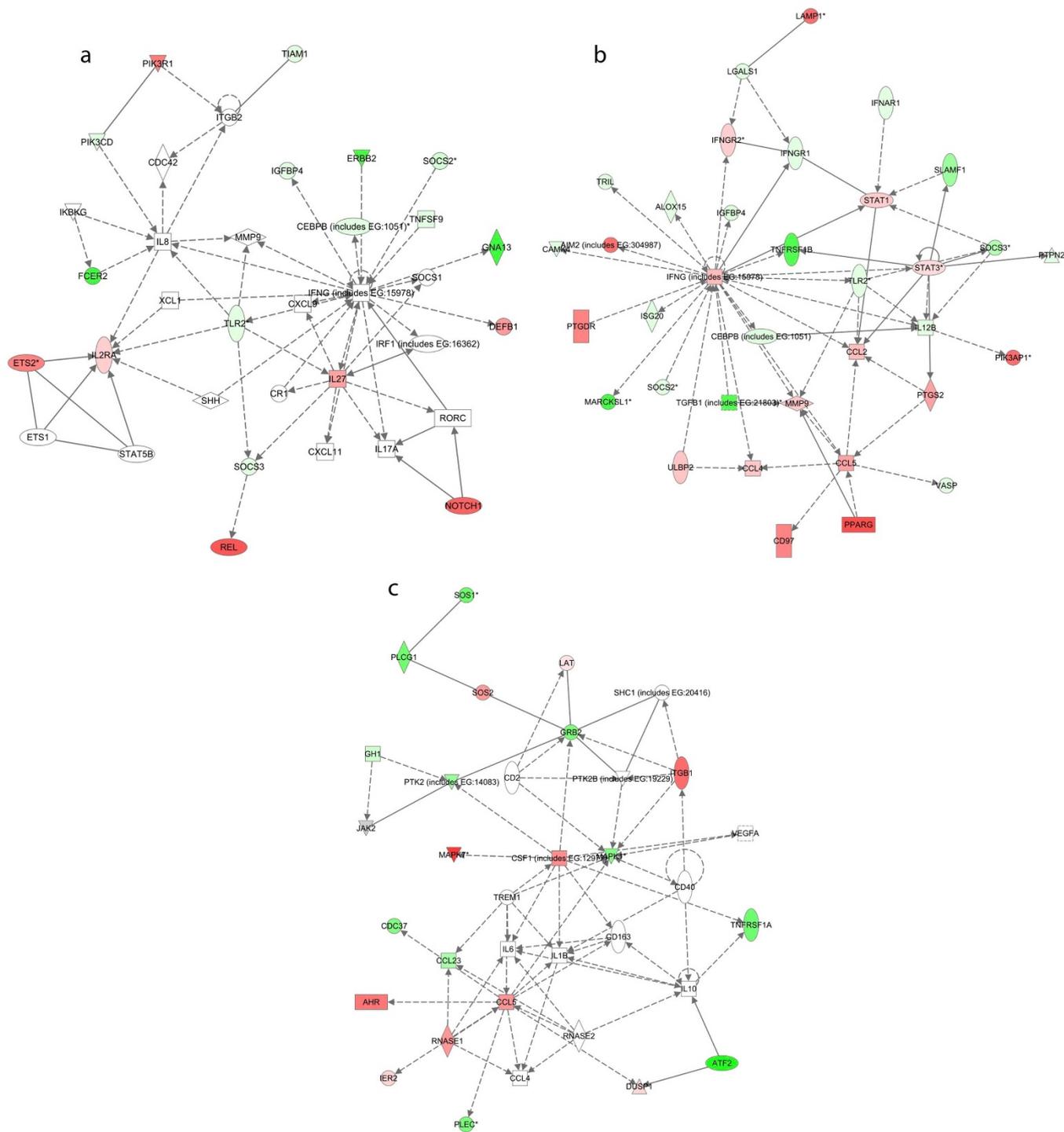
Macrophages are the primary cell type linked to obesity-mediated inflammation<sup>34</sup>. Recent studies have demonstrated that alveolar macrophages in adult obese asthmatics are of the pro-inflammatory phenotype that can be activated by leptin alone<sup>26</sup>. We found that PBMCs from obese non-asthmatics had evidence of decreased promoter methylation of several proteins including *ALOX15*, *IGFBP4*, *SOCS2* and *SOCS3* that are associated with obesity-specific activation of innate immune pathways<sup>38</sup>. However, in PBMCs from obese asthmatics, key cytokines and inflammatory mediators released by macrophages and involved in monocyte chemotaxis, and proliferation and survival including *CCL5* and *CSF1*, and downstream molecules such as *PI3K* involved in T cell differentiation<sup>39</sup>, signal transduction, and NF $\kappa$ B pathway<sup>40</sup> were hypomethylated compared to obese non-asthmatics, and healthy controls, together supporting a greater role of macrophage-mediated inflammation in childhood obesity-associated asthma, relative to children with obesity alone and healthy controls. Given that obesity-mediated inflammation is primarily driven by leptin<sup>11</sup> and leptin levels in our cohort were higher among obese asthmatics than obese non-asthmatics<sup>15</sup>, we speculate that DNA methylation may be one mechanism by which leptin may be influencing macrophage function and systemic inflammation among obese asthmatics. In keeping with the complex

biological pathways influenced by methylation, while systemic Th1 polarization was observed in obese asthmatic children<sup>15</sup>, IFN $\gamma$  promoter itself was not differentially methylated in obese asthmatics compared to normal-weight asthmatics. We observed that other differentially methylated molecules indirectly related with IFN $\gamma$  promoter and maybe altered the IFN $\gamma$  pathway but the extent to which these potentially played a role in the Th1 polarization by modulating IFN $\gamma$  production needs further investigation.

This study has a small sample size and only represents a first attempt to determine whether epigenetic changes are involved in obesity-associated asthma, with limited power to detect anything but the strongest and most consistent changes. Furthermore, we recognize that our use of mixed PBMCs, comprised of T cells, B cells and monocytes, reduces our ability to detect T cell-specific changes. To have generated data indicative of systematic changes occurring at loci with properties consistent with a role in asthma and obesity indicates that this avenue is worth pursuing further, and that epigenome-wide DNA methylation analysis can identify additional genes which may orchestrate the observed inflammatory patterns needs additional investigation. These results suggest that DNA methylation influences several genes that together determine the phenotype associated with a particular disease. As recently highlighted<sup>41</sup>, identification of a pathway in addition to methylation differences at a single gene level is needed to better define the pathophysiologic mechanisms associated with pulmonary disease and identify key biomarkers that may be amenable to pharmacologic intervention, given the reversible nature of DNA methylation<sup>42</sup>. We also recognize the lack of gene expression studies, due to limited number of PBMCs,



**Figure 3** | (a). Canonical pathways differentially methylated between obese asthmatics and normal-weight asthmatics. T cell and macrophage signaling and macrophage activation were the primary canonical pathways differentially methylated between obese and normal-weight asthmatics. (b). Canonical pathways differentially methylated between obese asthmatics and obese non-asthmatics. Macrophage activation and T cell signaling were the primary canonical pathways differentially methylated between obese asthmatics and obese non-asthmatics. (c). Canonical pathways differentially methylated between obese asthmatics and healthy controls. Macrophage activation, T cell signaling and IL-15 mediated natural killer (NK) cell modulated Th cell activation were the primary canonical pathways differentially methylated between obese asthmatics and healthy controls.



**Figure 4** | (a). Network of differentially methylated molecules in obese asthmatics compared to normal-weight asthmatics. Genes encoding for molecules in red nodes are hypomethylated in obese asthmatics while those in green nodes are hypermethylated relative to normal weight asthmatics. (b). Network of differentially methylated molecules in obese asthmatics compared to obese non-asthmatics. Genes encoding for molecules in red are hypomethylated in obese asthmatics while those in green are hypermethylated relative to obese non-asthmatics. The nodal relationships indicated in solid lines denote direct while those in dashed lines denote indirect interactions. The shape of each node denotes the molecule class: horizontal ovals are transcription factors, vertical ovals are transmembrane receptors, horizontal diamonds are peptidases, vertical diamonds are enzymes, squares are growth factors, horizontal rectangles are nuclear receptors, vertical rectangles are ion channels, inverted triangles are kinases, and circles are “other” molecules. (c). Network of differentially methylated molecules in obese asthmatics compared to healthy controls. Genes encoding for molecules in red are hypomethylated in obese asthmatics while those in green are hypermethylated relative to healthy controls. The nodal relationships indicated in solid lines denote direct while those in dashed lines denote indirect interactions. The shape of each node denotes the molecule class: horizontal ovals are transcription factors, vertical ovals are transmembrane receptors, horizontal diamonds are peptidases, vertical diamonds are enzymes, squares are growth factors, horizontal rectangles are nuclear receptors, vertical rectangles are ion channels, inverted triangles are kinases, and circles are “other” molecules.



limits the extrapolation of our results into biological consequences. However, since we had previously observed a Th1 polarized phenotype in the same samples that underwent quantification of DNA methylation, our current results provide a foundation for potential role of DNA methylation in Th1 polarization in obese asthmatics, and provide pilot data for future studies to validate our findings with gene expression studies. Given our small sample size, our results are also susceptible to by-chance findings. Hence, future studies of DNA methylation patterns of individual cell types combined with gene expression studies within the peripheral blood mononuclear cells are needed to better determine the role of methylation in the differentiation and activation of each immune cell in obese asthmatics.

In conclusion, in a pilot study investigating DNA methylation patterns among obese asthmatic pre-adolescent children compared to those with asthma alone, obesity alone and healthy controls, we found that methylation patterns in peripheral blood mononuclear cells in obesity-associated asthma are distinct and are indicative of activation of innate and adaptive immune responses, including T cell differentiation, and release of pro-inflammatory substances from macrophages. Although both obesity and obesity-associated asthma have similar inflammatory profiles with systemic non-atopic inflammation, the DNA methylation patterns suggest inherent differences with activation of distinct inflammatory pathways in obese asthmatics. Further studies are needed to investigate which cell types are contributing to the observed methylation patterns, and to confirm and validate our findings.

## Methods

**Study material and ethics statement.** Peripheral blood samples used to study genome-wide methylation were a subgroup of samples obtained from pre-adolescent minority children participating in a study investigating inflammatory patterns in obesity-associated asthma<sup>15</sup>. This subgroup consisted of samples from 32 children, including 8 from each of the 4 study groups of obese asthmatics, normal-weight asthmatics, obese non-asthmatics and healthy controls where the parent/legal guardian of the participating child had given written informed consent for use of the residual PBMCs for future studies. As previously described, obesity was defined as a body mass index (BMI) greater than 95th percentile for age and normal-weight as BMI less than the 85th percentile<sup>15</sup>. Asthmatics were those diagnosed by their primary care physicians, as per the National Asthma Education and Prevention Program (NAEPP) guidelines<sup>43</sup>. Co-existence of atopic conditions including allergic rhinitis and eczema did not differ between the two study groups with asthma. The characteristics of the subgroup including demographics and asthma severity did not differ from those of the parent study. The Institutional Review Board at Montefiore Medical Center approved the parent study as well as the use of residual de-identified samples for the current study.

The Ficoll-Hypaque method was used to isolate peripheral blood mononuclear cells (PBMCs) from whole blood, the purity of which was confirmed by flow cytometry [Figure S1]. The cells were aliquoted, frozen in 10% DMSO, and stored in liquid nitrogen until further processing. Genomic DNA was isolated from aliquots of  $3 \times 10^6$  PBMCs using Gentra Puregene Blood kit (Qiagen, Chatsworth, CA) following the manufacturer's instructions.

**HELP-tagging assay to test DNA methylation.** Epigenome-wide methylation studies in PBMCs from the four study groups were performed by using the massively-parallel sequencing (MPS)-based HELP-tagging assay on the genomic DNA as previously reported<sup>44,45</sup>. Briefly, 5  $\mu$ g of genomic DNA was digested by HpaII and purified by phenol/chloroform extraction followed by ethanol precipitation. The digested DNA was then ligated at the cohesive end to the first adaptor including a restriction enzyme site that was digested by EcoP151 followed by ligation of the second adaptor that was MPS compatible. These adaptors served as priming sites for ligation-mediated PCR amplification. Further, to generate longer flanking sequences, a T7 polymerase was added followed by a reverse transcription step, allowing the generation of libraries for MPS that were compared to human MspI reference libraries. Libraries were sequenced using Illumina Genome GAIIX/HiSeq 2000 sequencer following the manufacturer's instructions.

While MspI digestion is methylation insensitive and cuts the DNA at all CCGG sites, HpaII digestion is methylation sensitive, and cuts DNA only at CCGG sites where the central CG is unmethylated at the cytosine nucleotide. Comparison of HpaII count to MspI count for each locus thereby allows quantification of methylation. The angle obtained by plotting HpaII count on the y-axis and MspI count on the x-axis provides a quantitative measure of locus-specific methylation. A higher angle formed when HpaII count is high relative to MspI is evidence of relative hypomethylation while a smaller angle is evidence of hypermethylation<sup>19</sup>. Prior studies in our lab have demonstrated a correlation of  $\sim 0.9$  between replicates and between

angle values obtained by HELP-tagging and quantification of methylation by bisulphite MassArray (Sequenom)<sup>19,28,29</sup>.

**Library quality assessment.** As described by Jing et al.<sup>45</sup>, the Illumina genome analyzer uses the CASAVA software to perform image analysis and base calling, the outputs from which are the raw sequence reads in Illumina's QSEQ format. For quality assessment of the sequences, each noncallable (unknown) base represented as a period is converted to "N", which are statistically analyzed to assess sequencing success and individual cycle efficacy. The library quality is further assessed by determining the proportion and position of the 3' adaptor sequence within the reads, where the first two CG represent the cohesive end for ligation of HpaII digestion products, and the other six nucleotides are the EcoP151 recognition site. A high percent of reads containing the adaptor sequence starting at position 27 is indicative of a high quality library. By removing sequences with excessive tracts of unknown bases ("N"s) and those lacking the adaptor sequence, spurious sequences are eliminated. Of the remaining sequences, the first base is removed since it originated from the 3' adaptor ligation and the 5' adaptor is masked by replacing the bases with "N"s to maintain a uniform tag size. Illumina's alignment algorithm, ELAND, treats unknown ("N") bases as wild cards and therefore does not penalize them during alignment to the reference genome. During the alignment, a maximum of two mismatches are allowed and insertion/deletions are ignored. Statistics generated from the alignment include details on rejection of sequences that had greater than 10 aligned hits and those with no match at all, retaining only those that had unique or fewer than 10 aligned hits that were mapped to an annotated HpaII site. The counts of sequence tags aligned adjacent to every HpaII/MspI site are assessed. Tags that align to more than one locus are given a proportional count. Cumulative HpaII counts are normalized with MspI counts for each HpaII/MspI annotated site. To quantify methylation, the normalized accumulative proportional (NAP) count for the HpaII digested sample and the MspI reference sample are represented as Cartesian coordinates for vectors projected in two dimensional space where the Y axis represents the HpaII NAP count and the X-axis represents the MspI NAP count. Represented as a vector, the direction (angle relative to the origin) corresponds to quantification of hypomethylation. These angle values are then linear scaled to a range from 0 to 100 and stored as WIG files<sup>45</sup>. On an average, methylation status of  $1.8 \times 10^6$  loci is obtained per sample.

**Data analysis.** Demographic characteristics of the study population were analyzed using STATA statistical software version 10 (StataCorp, College Station, TX). DNA methylation results were analyzed using R (version 2.12.0 (<http://www.R-project.org>)). The MPS output was pre-processed and underwent normalization for total count numbers as described above. The locus-specific angle obtained by plotting HpaII against MspI was compared between obese asthmatics and normal weight asthmatics, obese non-asthmatics and healthy controls, using paired T-test. The small sample size in this pilot study does not allow us to set a False Discovery Rate, or adjust for multiple testing. We therefore analyzed the biological significance of the top  $2 \times 10^4$  or the top 1% most consistently differentially methylated loci in obese asthmatics compared to normal-weight asthmatics, obese non-asthmatics and healthy controls in which differential methylation were defined as an absolute difference of angle value of  $>20$ , corresponding to  $\sim 20\%$  difference in methylation. We report the locus-specific differential methylation in obese asthmatics compared to the other three study groups. Differentially methylated loci were defined in terms of genomic context (e.g., occurring in a gene promoter, defined as  $\pm 1000$  base pairs from the transcription start site). All p values reported are two tailed with statistical significance set *a priori* at 0.05. All data are available through the Gene Expression Omnibus database (accession number GEO:GSE43705).

**Ingenuity pathway analysis.** The biologic function application of Ingenuity Pathway Analysis software (IPA, [www.ingenuity.com](http://www.ingenuity.com), Redwood City, CA) was used to investigate whether differentially methylated gene promoters were enriched for specific functional relationships. The analysis was filtered to human genes and to immune cells. Canonical pathway identification was filtered to those associated with cellular or humoral immune response, or cytokine signaling. Differentially methylated gene promoters in obese asthmatic PBMCs associated most strongly with functional networks relevant to cell to cell signaling when compared to normal weight asthmatics, chemotaxis of natural killer (NK) cells when compared to obese non-asthmatic and differentiation of T lymphocytes when compared to healthy control PBMCs. Graphic representation of the canonical pathways and the functional networks was obtained from IPA.

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## Author contributions

D.R. conceived the project, conducted the experiments and wrote the manuscript. M.S. helped with the experiments, and with data analysis and critical review of manuscript. J.M.G. provided input on project conception, analysis of results and manuscript development.

## Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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