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1 Multi-Method Molecular Characterisation of Human Dust-Mite-associated Allergic
2 Asthma
3
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10 ## Abstract

11 Asthma is a chronic inflammatory disorder of the airways. Disease presentation
12 varies greatly in terms of cause, development, severity, and response to medication,
13 and thus the condition has been subdivided into a number of asthma phenotypes.
14 There is still an unmet need for the identification of phenotype-specific markers and
15 accompanying molecular tools that facilitate the classification of asthma phenotype.
16 To this end, we utilised a range of molecular tools to characterise a well-defined
17 group of adults with poorly controlled asthma associated with house dust mite (HDM)
18 allergy, relative to non-asthmatic control subjects. Circulating messenger RNA
19 (mRNA) and microRNA (miRNA) were sequenced and quantified, and a differential
20 expression analysis of the two RNA populations performed to determine how gene
21 expression and regulation varied in the disease state. Further, a number of
22 circulating proteins *(IL-4, 5, 10, 17A, Eotaxin, GM-CSF, IFNy, MCP-1, TARC, TNFa,*
23 *Total IgE, and Endotoxin)* were quantified to determine whether the protein profiles
24 differed significantly dependent on disease state. Finally, assessment of the
25 circulating "blood microbiome" was performed using 16S rRNA amplification and
26 sequencing. Asthmatic subjects displayed a range of significant alterations to
27 circulating gene expression and regulation, relative to healthy control subjects, that
28 may influence systemic immune activity. Notably, several circulating mRNAs were
29 detected in the plasma in a condition-specific manner, and many more were found to
30 be expressed at altered levels. Proteomic analysis revealed increased levels of
31 inflammatory proteins within the serum, and decreased levels of the bacterial
32 endotoxin protein in the asthma state. Comparison of blood microbiome composition
33 revealed a significant increase in the Firmicutes phylum with asthma that was
34 associated with a concomitant reduction in the Proteobacteria phylum. This study
35 provides a valuable insight into the systemic changes evident in the HDM-associated
36 asthma, identifies a range of molecules that are present in the circulation in a
37 condition-specific manner (with clear biomarker potential), and highlights a range of
38 hypotheses for further study.

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40 # Introduction

41

42 Asthma is a chronic inflammatory disorder of the airways and is a global public
43 health concern due to increasing prevalence and mortality rates (1–4). The World
44 Health Organisation has estimated that 300 million people are living with asthma,
45 and that 250,000 individuals die prematurely each year as a result of the disease (5).

46 Asthma can develop during childhood (early-onset) or in adulthood (late-onset) and
47 is characterised by chronic inflammation of the airways and intermittent episodes of
48 reversible airway obstruction (6,7). Over time, chronic inflammation of the airways
49 results in airway hyper-responsiveness and structural changes, including airway
50 fibrosis, goblet cell hyperplasia, increased smooth muscle mass, and increased
51 angiogenesis (7,8).

52 The causes of asthma are multifactorial, and include a complex variety of
53 environmental, immunological, and host genetic factors (7,9–13). Disease typically
54 occurs in genetically predisposed individuals (13,14), and clinical presentation is
55 highly heterogenous (15). Disease can vary greatly in terms of disease onset and
56 response to treatment (16). It can present as a chronic, stable disease, but also as
57 intermittent asthma exacerbations that can be fatal (17). Symptoms can be mild or
58 severe and arise as a result of a multitude of factors, including immunoglobulin-E
59 (IgE) mediated allergic responses, exposure to pollutants, exercise, stress, or airway
60 infections (17).

61 The complex nature of asthma pathogenesis has resulted in speculation as to
62 whether asthma is a single disease, or a spectrum of related diseases with subtle but
63 distinct differences in aetiology and pathophysiology (18,19). This has led to asthma
64 being separated into a number of phenotypes, which are then further subdivided into
65 several endotypes (6,15,18–20). These asthma phenotypes are triggered by
66 complex gene-environment interactions and respond differently to the various
67 asthma medications available. Individuals with eosinophilic asthma, for instance,
68 have been reported to have a good therapeutic response to inhaled or oral
69 corticosteroid therapy, whereas individuals with neutrophilic asthma have been found
70 to respond poorly to this therapeutic approach (21).

71 Diagnostic tools for identifying the various asthma phenotypes are limited, and thus
72 optimal treatment protocols are not being utilised in a number of patients. Moreover,
73 despite decades of research, there has been little progress in the development of
74 treatments since the introduction of inhaled ß2 adrenoceptor 2 selective agonists
75 (1969) and inhaled glucocorticosteroids (1974) (15). Long-term use of these
76 medications has been associated with a number of health concerns (22), including
77 the stunting of growth in children (23), cataract development (24,25), osteoporosis
78 (26,27), and cardiovascular events (28). Overall, an estimated 5-10% of asthmatics
79 fail to respond to conventional medications (29). In order to improve patient response
80 to treatment, and / or assist in the development of new therapeutics, an improved
81 knowledge of the molecular mechanisms that underlie the various asthma
82 phenotypes is required. Long-term, this may also facilitate the targeted use of
83 conventional asthma therapies, and facilitate the development of new medications

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84 aligned to the individual asthmatic phenotypes, subsequently reducing asthma
85 mortality and improving quality of life.

86

87 The focus of this study was to characterise, at the molecular level, a small but well-
88 defined cohort of patients with atopic asthma associated with house dust mite (HDM)
89 allergy. Global estimates suggest that 1-2% of the world's population are sensitive
90 to HDM (30), as are approximately 50% of asthmatic patients (30,31). HDM
91 sensitivity has been linked to increased asthma severity (32) and almost one-third of
92 patients with HDM sensitivity are unresponsive to current asthma therapies (33).
93 Increasing our understanding of this specific asthma phenotype is therefore crucial.
94 To this end we performed a comprehensive molecular characterisation of (1)
95 circulating mRNAs, (2) circulating microRNAs, (3) circulating protein-based markers
96 of the immune response and (4) integrated these data with our previous work
97 characterising evidence of a circulating microbiome.

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101 **Methods**

102 **Donor Population**

103 Atopic asthmatic individuals (n=5) with physician-diagnosed HDM allergy, and
104 gender and age-matched healthy control subjects (n=5) were recruited to the study
105 via SeraLabs Limited. Asthma patients were selected on the basis that they had
106 developed atopic asthma during early childhood and that their condition had
107 continued into adulthood and remained "poorly controlled". A full list of recruitment
108 criteria is presented in **(Table 1).**

109 Whole blood was drawn, following alcohol cleansing of the skin surface, into EDTA
110 containing tubes and stored on ice prior to centrifugation at 1000×*g* to obtain the
111 plasma component. All samples were analysed anonymously, and the authors
112 obtained ethical approval and written informed consent to utilise the samples for the
113 research reported herein.

114 The Independent Investigational Review Board Inc. ethically approved sample
115 collection by Sera Laboratories Limited from human donors giving informed written
116 consent. Furthermore, the authors obtained ethical approval from Keele University
117 Ethical Review Panel 3 for the study reported herein. All experiments were
118 performed in accordance with relevant guidelines and regulations.

119

120 **Table 1**: Donor population characteristics required for the study

121

Patient Criteria
• Have a BMI < 30
• Be a non-smoker
• Have been diagnosed with atopic asthma during childhood
• Have severe/ poorly controlled asthma
• Must not be on any oral steroid treatment
• Must be allergic to the house dust mite
• Must not have diabetes, COPD, or hypertension

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Analysis of Inflammatory proteins

Plasma levels of interleukin (IL)-4, IL-5, IL-10, IL-13, IL-17A, IFNγ, TARC, Eotaxin, GM-CSF, MCP-1, RANTES, and TNFα, was determined using a qualitative enzyme-linked immunosorbent assay (ELISA) custom designed for this study. Two multi-analyte sandwich ELISAs (Qiagen) were used, and analysis of the inflammatory proteins was achieved using the recommended Multi-Analyte ELISArray kit protocol (QIAGEN). Statistical analysis was performed by carrying out a Shapiro-Wilk normality test and a Wilcox rank sum test using *R* software Version 3.5.0.

Quantitative analysis of total IgE

The concentration of total immunoglobulin E (IgE) was determined using sandwich ELISA (Genesis Diagnostics Ltd). The ELISA was performed in duplicate using the recommended protocol, and absorbance was measured at 450nm using an ELX800 absorbance reader (BioTek). Statistical analysis was performed by carrying out a Shapiro-Wilk normality test and an unpaired T test using *R* software Version 3.5.0.

Quantitative analysis of endotoxin concentration

Circulating bacterial endotoxin concentration was measured using a Pierce™ Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin quantitative kit (Thermo Scientific). The assay was performed in triplicate using the recommended protocol, and absorbance was measured at 450nm using an ELX800 Absorbance reader (BioTek). Statistical analysis was performed by carrying out a Shapiro-Wilk normality test and an unpaired T test using *R* software Version 3.5.0.

Total RNA extraction

Total RNA was extracted from 500μl of human plasma using the Qiagen serum and plasma miRNeasy kit. The quantity and quality of the RNA extracts was determined using the QuBit fluorimeter (Invitrogen) and BioAnalyzer (Agilent).

Library Preparation and Next Generation Sequencing

Messenger RNA (mRNA) sequencing libraries were prepared using the SMARTer Universal Low Input RNA kit, and sequenced (Illumina HiSeq 2000) with a paired-end 90 nucleotide read metric. Small RNA sequencing libraries were prepared using the TruSeq small RNA library kit (Illumina), and sequencing was conducted on the Illumina HiSeq 2000 platform.

Raw sequencing data were trimmed of sequencing adaptors and low-quality reads removed using the Trim Galore package – a wrapper that incorporates CutAdapt and FastQC. For whole transcriptome analysis, quality-controlled reads were aligned to the Human Genome build hg19 using TopHat, a splice-junction aware mapping utility

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161 necessary for the successful mapping of intron-spanning (multi-exon) transcripts.
162 Transcriptome assembly was performed using CuffLinks and a merged transcript
163 representation of all samples produced using CuffMerge. Transcripts expressed at
164 significantly different levels between the asthma and control samples were identified
165 using CuffDiff, with a Q value $\leq$ 0.05 considered significant (34). MicroRNA (miRNA)
166 analysis was performed by mapping miRNA reads to miRbase Version 21 using
167 sRNAtoolbox (35). Differential expression of the miRNA reads was determined
168 following statistical analysis with edgeR for R (36).

169

170 **Biological Pathway Analysis**

171 Biological functions of the mRNA and miRNA that were differentially expressed
172 between asthma and control subjects (defined as Q ≤ 0.05 in the mRNA dataset; and
173 FDR ≤ 0.05 in the miRNA dataset) were determined using Ingenuity Pathway
174 analysis (IPA) software.

175 Networks of genes comprising known biological processes were identified using IPA.
176 Causal inference analysis was then applied to determine upstream regulators that
177 may explain the pattern of differential expression seen. Casual inference analysis
178 involved the generation of an enrichment score (Fisher's exact test *P* value) and a Z
179 score to determine the possible upstream biological causes of the differential gene
180 expression observed in the asthmatic subjects (37). The enrichment score measured
181 the overlap of observed and predicted regulated gene sets, whilst the Z score
182 assessed the match of observed and predicted up/ down regulation patterns (37).
183 Putative regulators that scored an overlap P value ≤ 0.05 were deemed statistically
184 significant, and the Z scores were used to determine the activity of the putative
185 regulators (an upstream regulator with a Z score greater than 2.0 was considered
186 activated, whilst an upstream regulator with a Z score less than -2.0 was considered
187 deactivated). Causal inference analysis was also used to predict the downstream
188 effects the differentially expressed genes and miRNA could have on biological
189 processes and functions in the asthmatic subjects.

190

191 **Circulating microbiome analysis**

192 We have previously reported evidence of a circulating microbiome in the blood of
193 both asthmatic and healthy patients (38) using oligonucleotide primers reported in
194 (Supplementary Materials, S1). Here, we re-analysed this data with the aim of
195 identifying organisms that were differentially present or abundant dependent on
196 disease status. The QIIME pipeline was used for quality filtering of DNA sequences,
197 demultiplexing, and taxonomic assignment. Alpha diversity was determined by
198 calculating Shannon and Chao1 diversity indices. Differences in relative abundance
199 was calculated by performing Shapiro-Wilk normality tests and the appropriate
200 statistical test (unpaired T tests when the samples displayed gaussian distribution
201 and Wilcox rank sum test when the samples did not display Gaussian distribution) on
202 bacterial abundance data (read counts normalised to the total number of bacterial
203 reads per patient) using *R* software Version 3.5.0.

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204 In addition to standard statistical tests, the linear discriminant analysis effect size
205 (LefSe) method was used to identify the bacterial taxa most likely to explain the
206 differences in microbial populations present in the asthmatic cohort compared to the
207 control cohort. In brief, the non-parametric factorial Kruskal-Wallis sum-rank test was
208 applied to the 16S relative abundance data in order to detect features with significant
209 differential abundance in the asthmatic cohort compared to the control group. A set
210 of pairwise tests among subclasses using the unpaired Wilcoxon rank-sum test were
211 then carried out to assess whether the detected differences in relative abundance
212 were consistent with respect to biological behaviour. Linear discriminant analysis
213 (LDA) was then performed to predict the effect of each identified differentially
214 abundant bacterial taxa.

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217 **Results**

218 **Patient Recruitment and Characterisation**

219 Five female asthmatic subjects were recruited in accordance with the inclusion
220 criteria detailed in (**Table 1**). The mean age of the asthmatic subjects was 39.6 ±
221 11.7 years, and all had been clinically diagnosed with atopic asthma during early
222 childhood (mean age of onset = 6.2 ± 3.2 years) (**Table 2**). At the time of sample
223 collection, the asthmatic subjects were on prophylactic therapy to minimise the
224 occurrence of disease symptoms (see Supplemental Material, S2). Asthma severity
225 was determined using the internationally recognised Asthma Control Questionnaire
226 (ACQ) (39,40), and all the asthmatic subjects scored a total ≥ 10.0 (mean total score
227 = 10.8 ± 0.75) (see Supplemental Material, S2). Additionally, three of the asthmatic
228 subjects were clinically diagnosed with other atopic diseases, including allergic
229 rhinitis, allergic dermatitis, and nasal polyps (see Supplemental Material, S2).

230 Five non-asthmatic females with a mean BMI of 24.3 ± 2.1 were recruited to the
231 study as healthy controls. The control subjects had never smoked and had a mean
232 age of 39.4 ±10.3 years (**Table 2**). Two of the controls, Control_2 and Control_3,
233 reported self-diagnosed dermatitis, although neither had received diagnosis by a
234 physician for this condition.

235

236 **Table 2:** Characterisation of the asthmatic (n = 5) and control subjects (n = 5) at the
237 time of sample collection. S.D. = standard deviation

238

Characteristic	Allergic Asthmatics	Non-Asthmatics
Demographic characteristics		
Age - yr		
Mean (S.D)	39.6 (11.7)	39.4 (10.3)
Range	19 - 52	23 - 49
Race or ethnic group – no. (%)		
Caucasian	2 (40)	2 (40)
Hispanic	3 (60)	3 (60)
Sex – no. (%)		
Female	5 (100)	5 (100)
Male	0 (0)	0 (0)
Smoking History		
Smoking Status – no (%)		
Never Smoked	5 (100)	5 (100)
Former Smoker	0 (0)	0 (0)
Smoker	0 (0)	0 (0)
BMI		
Mean (S.D)	24.4 (2.6)	24.3 (2.1)
Range	21.5 – 27.8	21 – 26.4

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Inflammatory proteins

To determine the immune status of the asthmatic patients at the time of sample collection, characterisation of various chemokines and cytokines associated with asthma pathology was performed.

Qualitative ELISA was performed on the blood samples in order to profile the inflammatory state of the asthmatic and control, and inflammatory proteins under investigation included interleukin (IL)-4, IL-5, IL-10, IL-13, IL-17A, eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN□), monocyte chemoattractant protein 1 (MCP-1), thymus and activation regulated chemokine (TARC), and tumour necrosis factor alpha (TNFA). Additionally, the concentration of the pro-inflammatory bacterial endotoxin protein was measured, and total IgE present in the blood was quantified to determine the atopic state of the asthmatic subjects.

With regards to the host-derived inflammatory proteins, 10 out of the 12 inflammatory proteins under investigation were detected in the blood samples (see Supplementary Materials, S3).

Overall the asthmatic subjects were found to have elevated levels of inflammatory proteins compared to the controls, as determined by increased levels of all inflammatory proteins examined. This was particularly apparent for chemokines TARC (Fold change = 4.173; P value = 0.095), GM-CSF (Fold change = 3.607; P value = 0.111), and IFN□ (Fold change = 20.871; P value = 0.195) (**Figure 1A, B, and C**). However, it should be noted that there were no statistically significant increases detected for any of the individual proteins. This was likely due to the asthmatic subjects having a greater level of diversity with regards to inflammatory protein levels compared to the control subjects (**Figure 1**).

Of interest was the levels of IL-17A observed. This protein whilst not significantly increased in the asthmatic subjects (P value = 0.413), was found to be present at higher levels in asthmatic subjects who suffered additional atopic complications (Asthma_1, Asthma_2, and Asthma_4) and the two control subjects who had self-reported atopic dermatitis (Control_2 and Control_3) (see Supplementary Material S2 and S3). This suggests that whilst systemic levels of this cytokine are not elevated in asthma, IL-17A levels may be elevated in the blood of individuals with other atopic conditions, such as allergic rhinitis and atopic dermatitis.

Moreover, the asthmatic cohort appeared divided with regards to the inflammatory protein profiles, whereby asthmatic subjects Asthma_2 and Asthma_4 typically had high levels of circulatory inflammatory proteins, whilst asthmatic subjects Asthma_1, Asthma_3 and Asthma_5 displayed protein levels similar to those observed for the control subjects. This is reflective of the heterogenous nature of asthma pathology and suggests that possibility of asthma sub-phenotypes that display varying levels of circulatory inflammatory proteins. We comment upon this heterogeneity, and the impact of this upon sample size selection in the concluding section.

Total IgE was detected in 50% of the blood samples under investigation (three control subjects and two asthmatic subjects (**Figure 1D**).

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282 For the purpose of statistical analysis, samples with undetectable levels of IgE were
283 given an IgE concentration value of 0. Comparison between the concentrations of
284 IgE detected in the asthmatic samples compared to the control samples revealed no
285 significant differences. This is likely due to the small number of samples with
286 detectable IgE. However, samples Asthma_2 and Asthma_4 again had notably
287 higher levels that the rest of the sample set. Within the asthmatic cohort it was these
288 two subjects that had the highest levels of inflammatory proteins under investigation
289 (see Supplementary Materials, S3), and thus the results of IgE quantification further
290 support the concept of asthma sub-phenotypes with different circulatory immune
291 status. As noted previously, such hypotheses require investigation with a much
292 larger study cohort.

293 Overall, endotoxin levels were found to be reduced in the asthmatic subjects (**Figure**
294 **1E**; P value = 0.0650). Within the asthma cohort, subjects with additional atopic
295 complications (i.e. allergic rhinitis, allergic dermatitis) displayed lower endotoxin
296 concentrations compared to the asthmatic subjects that did not have additional
297 atopic complications. This finding was further supported by the observation that
298 within the control cohort, subjects with previously reported atopic dermatitis
299 displayed circulatory endotoxin concentrations similar (i.e. lower than those subjects
300 reporting no atopic conditions) to those observed in the asthma cohort.

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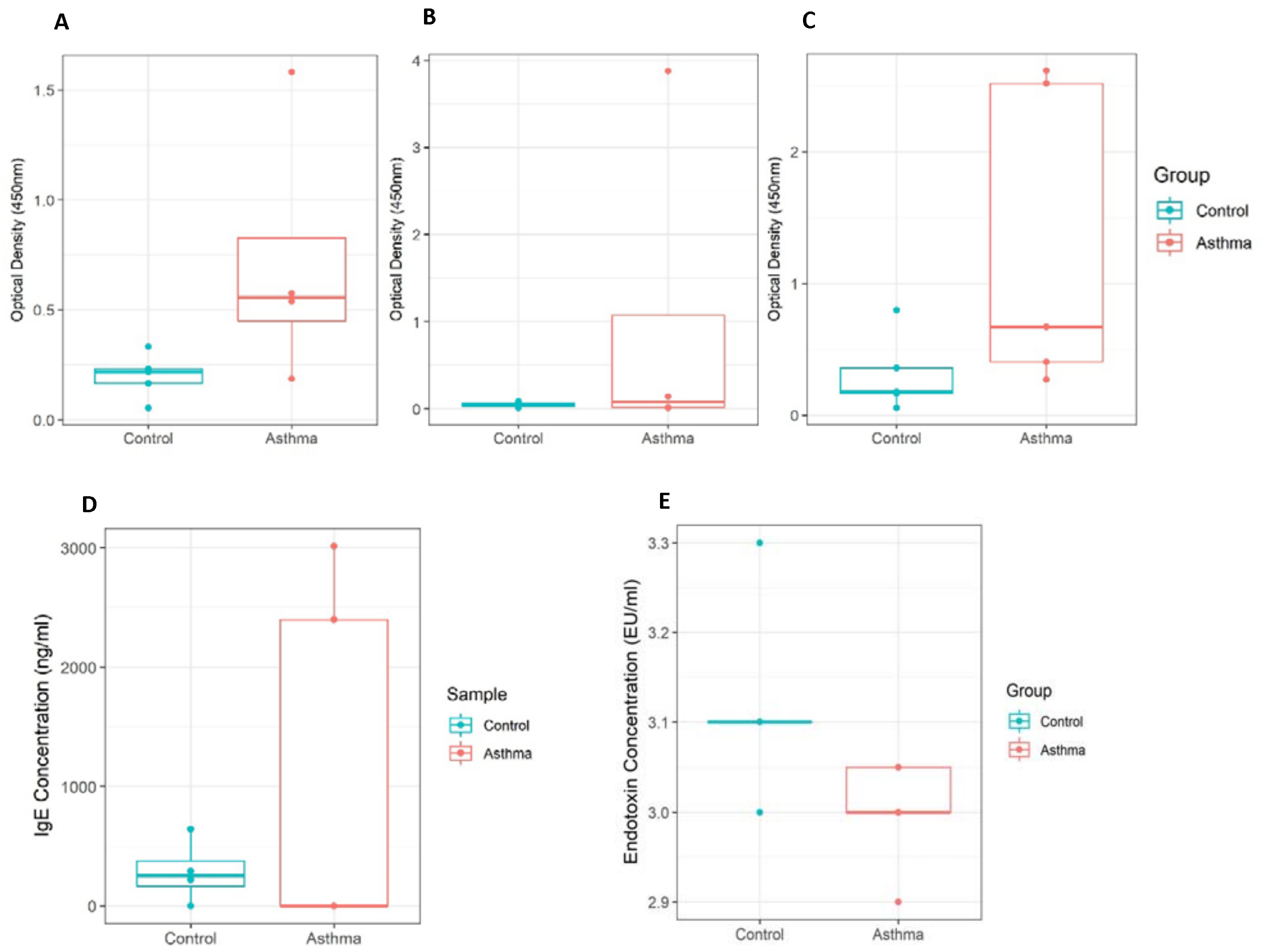

304

305 **Figure 1: Analysis of circulatory inflammatory proteins present in blood**
306 **samples from control subjects (n = 5) and asthma subjects (n = 5). A =** levels of
307 GM-CSF present in the blood of asthmatic subjects (n = 5) and control subjects (n =
308 5) using qualitative ELISA analysis, P value = 0.111 (Wilcoxon rank sum test with
309 continuity correction); **B =** levels of IFN present in the blood of asthmatic subjects
310 (n = 5) and control subjects (n = 5) using qualitative ELISA analysis, P value = 0.195
311 (Wilcoxon rank sum test with continuity correction); **C =** levels of TARC in the blood
312 using of asthmatic subjects (n = 5) and control subjects (n = 5) qualitative ELISA
313 analysis, P value = 0.095 (Wilcoxon rank sum test with continuity correction); **D =**
314 Concentrations of total IgE protein present in the blood of asthmatic subjects (n = 4)
315 and control subjects (n = 5) using quantitative ELISA analysis, P value = 1.0
316 (Wilcoxon rank sum test with continuity correction); **E =** Concentrations of bacterial
317 endotoxin present in the blood of asthmatic subjects (n = 5) and control subjects (n =
318 5) using Limulus Amebocyte Lysate (LAL) Chromogenic quantification. P value =
319 0.0650 (unpaired T test). EU/ml = endotoxin units per millilitre. Data points at 3.1
320 EU/ml for control = 3; Data points at 3.0 EU/ml for asthma = 2; Data points at 3.05
321 EU/ml for asthma = 2.

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322 **mRNA Sequencing and Differential Expression Analysis**

323 Approximately 20,000,000 messenger RNA (mRNA) read pairs were generated from
324 each plasma sample (average 44,000,000 ± 3,100,000 reads), with no significant
325 differences in read count identified between the two cohorts.

326 Expression of a total of 14, 226 genes was detected through assessment of the
327 circulating transcriptome (i.e. those RNAs present in the plasma). Given the nature
328 of our sample type, the extent of read mapping to key mRNAs was confirmed
329 visually by appraising the resulting BAM file against hg19 using IGV (data not
330 shown). Sample Asthma_2 failed to map satisfactorily to hg19 and was thus
331 excluded due to concerns this would induce bias into our downstream analyses.
332 Statistical analysis, as detailed previously, revealed 287 genes were differentially
333 expressed in the asthmatic subjects (as defined by a $Q \leq 0.05$ and a Log2 Fold
334 Change > 0.6). Within the asthmatic cohort, 90 of the differentially expressed genes
335 showed significantly increased expression, and 197 genes displayed significantly
336 decreased expression. Genes that displayed the highest degree of differential
337 expression within the asthmatic subjects are listed in **Table 3**. A full list of
338 differentially expressed genes can be viewed in the supplementary materials
339 (Supplementary Materials, S4)

340

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342 **Table 3: Genes that were most differentially expressed in the asthmatic**
343 **subjects (n = 4) compared to the control subjects (n = 5).** Where genes are
344 expressed in a condition-specific manner, Log2 fold change is replaced with "Control
345 Only" or "Asthma Only" as appropriate. Quantity of the gene is shown as Fragments
346 Per Kilobase of transcript per Million mapped (FPKM) reads

Gene	Control Mean	Asthma Mean	Fold Change (log2)	Q Value
Downregulated Genes				
DOHH	972.908	0	Control Only	0.002975
PTRH2	87.7907	0	Control Only	0.002975
C15orf41	79.1979	0	Control Only	0.002975
HIST1H3I	30.2331	0	Control Only	0.002975
HOXC10	26.4924	0	Control Only	0.002975
TSPYL5	18.9517	0	Control Only	0.002975
NFXL1	17.8423	0	Control Only	0.002975
RAB3IL1	15.1233	0	Control Only	0.002975
LINC00085	15.0233	0	Control Only	0.002975
ARV1	14.0641	0	Control Only	0.002975
Upregulated Genes				
HIST1H3C	0	90.5782	Asthma Only	0.002975
HDAC9	0.731644	52.1632	6.15575	0.005217
PRAM1	0	3.05743	Asthma Only	0.005217
PML	0.948462	178.238	7.554	0.007164
RAB6B	0	8.90346	Asthma Only	0.007164
NRP1	0.92425	18.8945	4.35354	0.010799
CD93	0	14.3366	Asthma Only	0.010799
GPR56	1.86976	98.5377	5.71975	0.012559
MR1	1.07632	17.8916	4.0551	0.017952
TOP1MT	0.344555	59.0342	7.42067	0.017952

347

348 Interestingly, there were numerous genes that were expressed in a condition specific
349 manner (96 genes were uniquely expressed in the control subjects, and 64 genes
350 were uniquely expressed in the asthmatic subjects). To determine whether the
351 asthmatic subjects had a distinct gene expression profile compared to the control
352 subjects, genes that displayed robust levels of expression (as determined by a mean
353 LOG2 FPKM score $\geq$ 6.0) were plotted as a heatmap and unsupervised cluster
354 analysis was performed using Euclidean distance (**Figure 2**).

355

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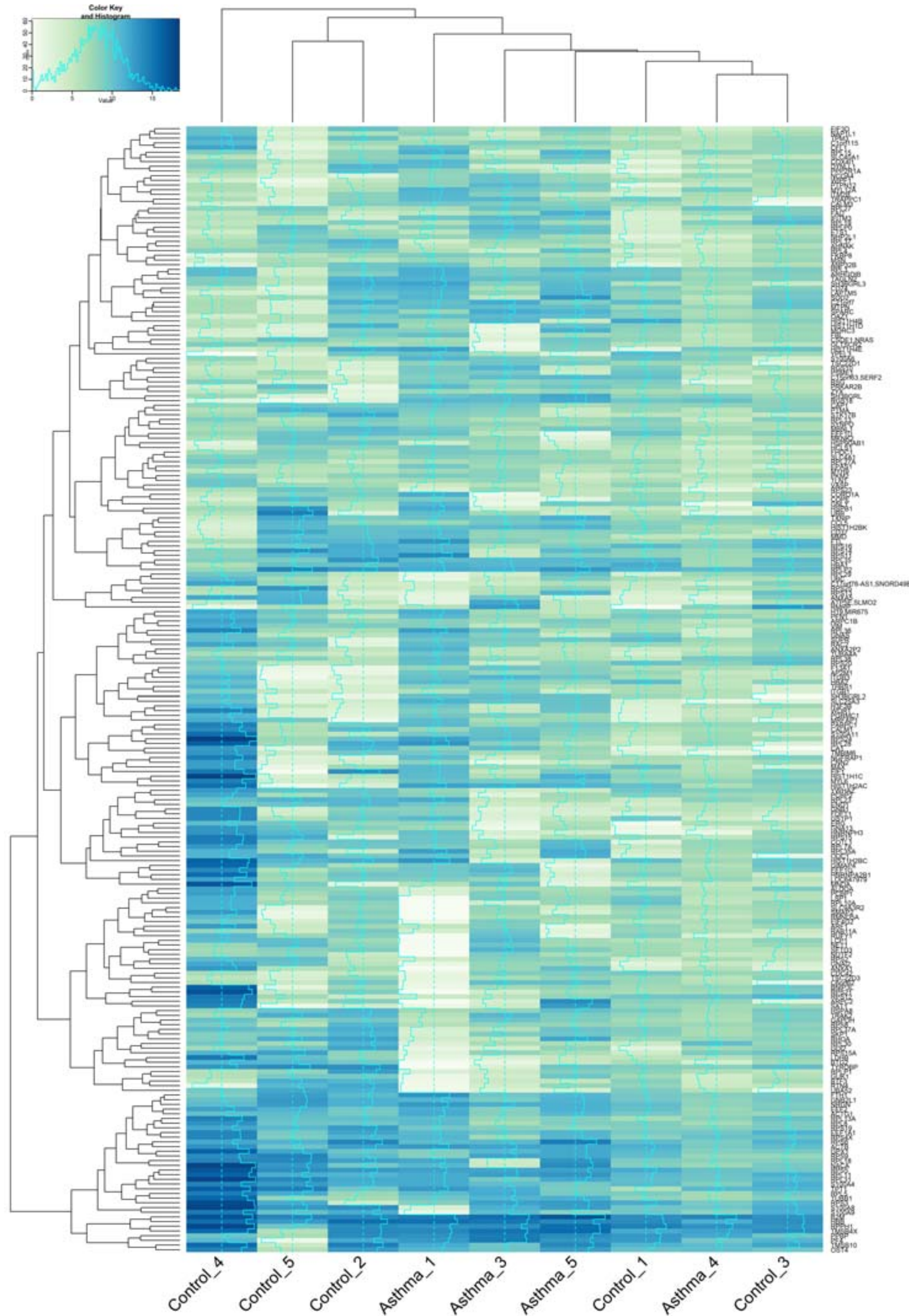

Figure 2: Heatmap showing highly expressed genes in control subjects (n = 5) and asthma subjects (n = 4). Gene expression is determined by quantification of circulatory mRNA present in the plasma samples and is expressed as log2 normalised Fragments Per Kilobase of transcript per Million mapped (FPKM) reads. Highly expressed genes, as determined by a mean log2 FPKM score $\geq$ 6.0 are

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364 plotted, and Cluster analysis (Euclidean distance) informs the X and Y-axis
365 dendrograms.

366 Cluster analysis revealed that subject Control_4 had a relatively unique mRNA
367 profile. For the remaining subjects, two clusters formed on the basis of circulatory
368 mRNA populations. Cluster 1 was formed of Control_5 and Control_2; and Cluster 2
369 was comprised of Asthma_1, Asthma_3, Asthma_5, Control_1, Asthma_4, and
370 Control_3. The dominance of asthmatic subjects in Cluster 2 suggests the possibility
371 of a distinct asthma mRNA profile that would likely be more apparent in a larger
372 sample group. Of interest, Asthma_4 clustered more closely with control Subjects
373 Control_1 and Control_3. This asthmatic subject was the youngest member of the
374 asthma cohort, with an age of 19 years, and the subject had been suffering from
375 asthma for just 14 years compared to the mean length of 38 years that our other
376 subjects had been living with the disease. It is tempting to speculate that asthmatic
377 mRNA profiles become more divergent from control profiles as the disease
378 progresses over time, however our sample size restricts further analysis of this.

379 The diversity of genes being expressed within the circulatory system was assessed
380 using principal coordinate analysis (PCA) (**Figure 3**).

381

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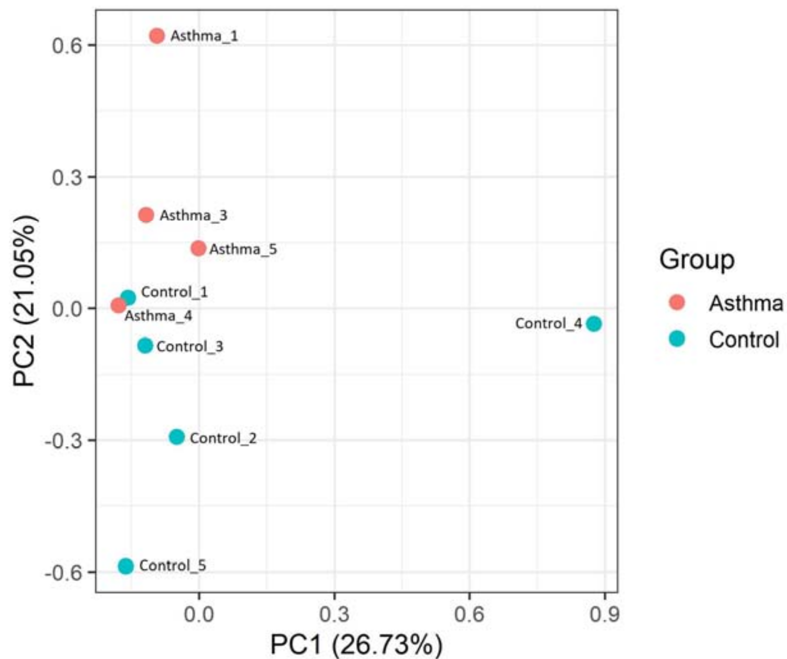

383

384 **Figure 3: Principal component analysis ordination of Bray Curtis dissimilarity**
385 **between circulatory mRNA populations present in control subjects (n = 5) and**
386 **asthma subjects (n = 4).** Principal component analysis was performed on a gene
387 population dataset using quantitative mRNA Fragments Per Kilobase of transcript
388 per Million mapped (FPKM) reads that had been normalised using log2. Only genes
389 with mean FPKM scores $\geq$ 6.0 were included in the dataset, and the principal
390 coordinate analysis was performed using Bray Curtis dissimilarity and R software.
391 Blue data points = Control; Orange datasets = Asthma

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397 Examination of Bray Curtis dissimilarity between the subjects found that,
398 unsurprisingly, samples clustered similarly to that observed using unsupervised
399 clustering (Euclidean distance). PCA analysis, did however reveal that the control
400 and asthmatic subjects were differentiated on the basis of principal component (PC)
401 2, whereby asthmatic subjects had a positive PC2 score and control subjects had a
402 negative PC2 score. Moreover, Asthma_4 clustered with the control subjects, thus
403 providing additional evidence that this subject has a mRNA profile similar to the
404 control subjects.

405

406 To determine whether differential gene expression could be linked to asthma
407 pathology, we compared the differentially expressed genes identified herein, to a
408 recently released database of genes associated with asthma pathology - AllerGAtlas,
409 2018 (41). Of the 287 genes identified as being significantly differentially expressed
410 in the asthmatic subjects, 8 genes were identified in the asthma gene database.
411 These genes included complement regulatory protein 46 (CD46), interleukin 7
412 receptor (IL7R), galactin 3(LGALS3), myeloperoxidase (MPO), neurotensin (NTS),
413 phosphodiesterase 4A (PDE4A), toll-like receptor (TLR) 1, and vitamin D receptor
414 (VDR). Four of the genes were upregulated in the asthmatic subjects (VDR, NTS,
415 TLR1, and MPO) and four were downregulated in the asthmatic subjects (LGAL3,
416 CD46, IL7R, and PDE4A) (**Table 4**). Moreover, gene expression was predominately
417 condition specific. Of the upregulated genes, NTS, TLR1, and MPO mRNA was only
418 detectable in the asthma samples, whilst in the downregulated genes, IL7R and
419 PDE4R mRNA was only observed in the control samples (see Supplementary
420 Materials, S4).

421

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Table 4: Genes with significant differential expression in the asthmatic subjects compared to control subjects that are associated with asthma pathology. Differential gene expression was determined using the Tuxedo protocol (Galaxy software) on log2 normalised mRNA Fragments Per Kilobase of transcript per Million mapped (FPKM) reads sequenced from plasma samples from asthma subjects (n = 4) and control subjects (n = 5). Gene function with regards to asthma pathology was determined using the asthma database AllerGAtlas, 2018 (41) and a general literature search using the relevant search engines.

Gene	Expression in Asthma	Function	Reference
CD46	Downregulated	Differentiation of IL-10 producing regulatory T cell type 1 cells Differentiation of Th1 cells Inhibition of HDM allergenic activity	(42)(43) (44)(45) (46)
IL7R	Downregulated	Marker for Treg activation T cell development Eosinophil survival	(47) (48)
LGALS3	Downregulated	Inhibition of IL-5 expression Inhibition of eosinophil and T cell infiltration Negative regulation of Th17 polarization	(49) (49) (50)
MPO	Upregulated	Initiation of lipid peroxidation	(51)
NTS	Upregulated	Mast cell degranulation	(52)(53)
PDE4A	Downregulated	Production of CD4+ T cell cytokines (IL-2, IL-4, IL-5, IFN[illegible]) Production of TNFα Production of leukotriene B4 Production of eotaxin Airway goblet cell hyperplasia	(54)(55)(56) (54) (54) (56) (56)
TLR1	Upregulated	Antimicrobial activity	(57)(58)(59)
VDR	Upregulated	Development of airway inflammation and hyperresponsiveness Eosinophilia Inhibits IgE production	(60) (60) (60)(61)

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433 The genes identified in the asthma gene database (41) were found to influence a
434 number of key components of asthma pathology, including eosinophil and T cell
435 migration, production of Th2 cytokines (IL-4, IL-5, and IL-13), mast cell
436 degranulation, IgE production, and airway hyperresponsiveness. Moreover, several
437 of the downregulated genes (CD46, IL7R), have been found to have roles in Treg
438 differentiation and activation. These cells are important regulators of T cell activity
439 (62–65) , and thus downregulation of CD46 and IL7R suggests loss of control of T
440 cell activity in the asthmatic subjects.

441

442

443 **miRNA Quantification**

444 Approximately 10,000,000 micro RNA (miRNA) reads were generated from each
445 plasma sample (range = 10,276,765 - 16,812,591, mean = 12,030,581 $\pm$ 1,911,104),
446 and there were no significant differences in read count identified between the control
447 and asthma samples.

448 Using miRanalyzer (35) and edgeR (36), we identified 166 known miRNAs present
449 in the plasma samples (**Figure 4**), which is consistent with previously reported
450 studies (66–70). To determine whether the asthmatic subjects had distinct miRNA
451 profiles compared to the control subjects, miRNA expression was plotted as a
452 heatmap, and unsupervised clustering was performed using Euclidean distance
453 (**Figure 4**).

454

455

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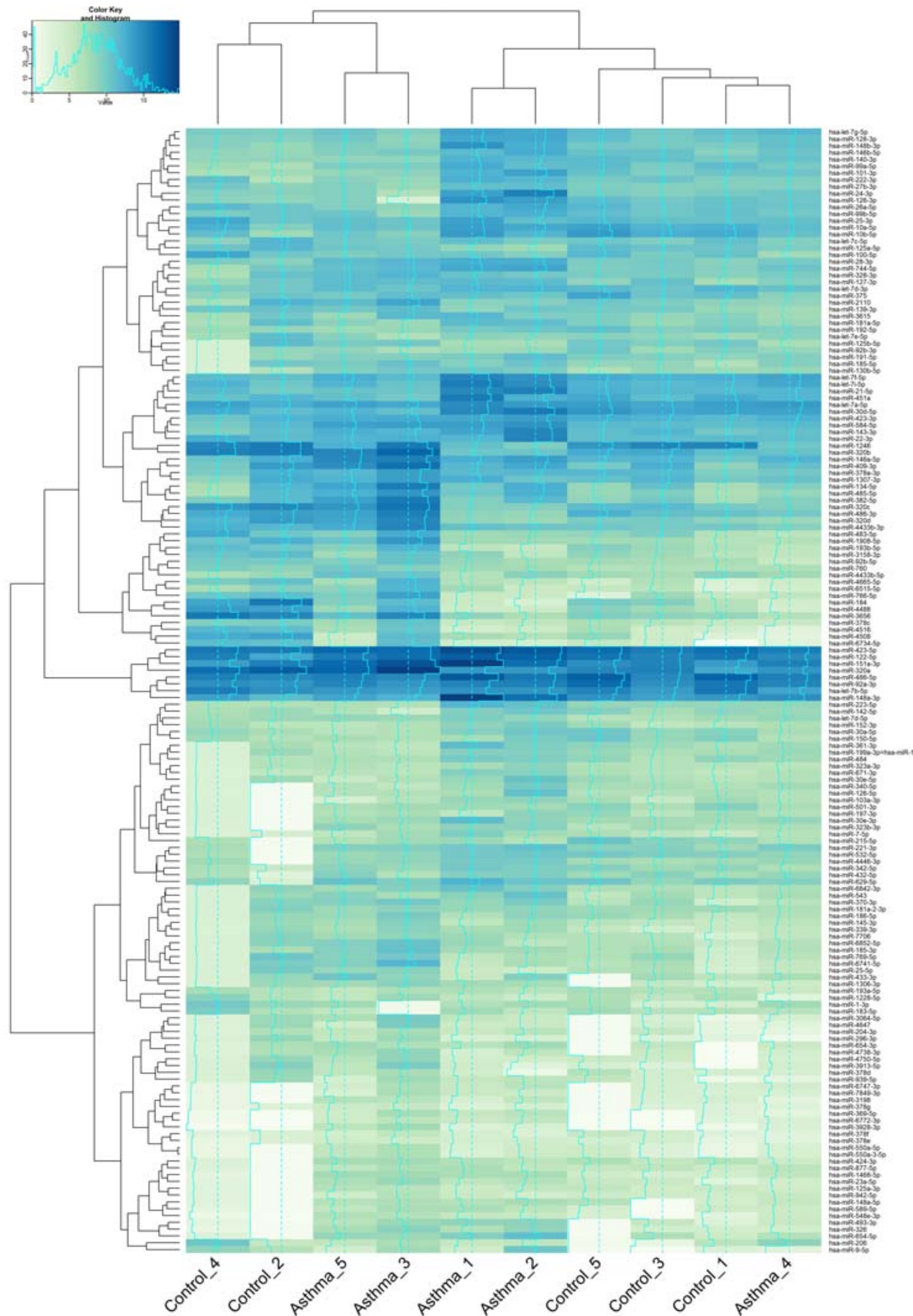

Figure 4. A Heatmap showing expression levels of circulatory miRNA in control subjects (n = 5) and asthmatic subjects (n = 5). miRNA expression is determined by quantification of circulatory miRNA detected in the plasma samples and is expressed as log2 normalised Counts per Million mapped (CPM) reads. Cluster analysis (Euclidean distance) informs the X and Y-axis dendrograms

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464 Analysis of miRNA expression revealed the presence of two clusters with regards to
465 the miRNA populations present within the plasma. Cluster 1 was composed
466 Control_4, Control_2, Asthma_5, and Asthma_3; and Cluster 2 was made up of
467 Asthma_1, Asthma_2, Control_5, Control_3, Control_1, and Asthma_4. Within each
468 cluster two sub-clusters formed, and each sub-cluster was formed of either control
469 subjects or asthma subjects. The one exception was Asthma_4, which clustered with
470 other control subjects.

471 Of interest, the two asthma sub-clusters that formed appeared to be governed by the
472 presence or absence of additional atopic complications. Asthma_5 and Asthma_3
473 clustered together and both subjects were free of additional atopic complication,
474 whereas Asthma_1 and Asthma_2 clustered together, and both subjects had
475 additional atopic complications such as allergic rhinitis. As we have noted previously,
476 further study using a larger asthma cohort would be required to determine this
477 association given the clear heterogeneity noted.

478 Statistical analysis revealed that 13 miRNAs were differentially expressed (defined
479 as FDR P value $\leq$ 0.05 and a fold change $\geq$ 2.0) in the asthmatic subjects compared
480 to the control subjects (**Figure 5**, see also Supplementary Materials S5). As
481 predicted, Asthma_4 displayed miRNA levels similar to those observed in the control
482 subjects. As stated previously, Asthma_4 was the youngest of the asthmatic subjects
483 and had been living with the disease for the shortest period of time. It is tempting to
484 speculate that asthmatic miRNA profiles become more divergent from control
485 subjects as the disease progresses over time, and that this in turn alters gene
486 expression.

487

488

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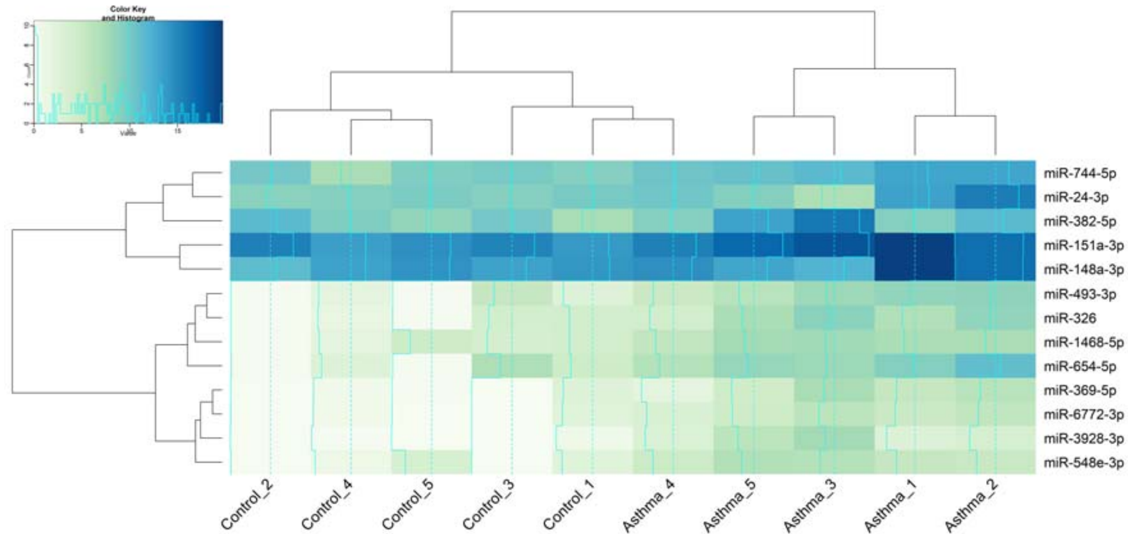

490

491

492 **Figure 5: A heatmap showing expression levels of circulatory miRNA that**
493 **displayed significant differential expression in asthmatic subjects (n = 5)**
494 **compared to control subjects (n = 5).** miRNA expression was determined by
495 quantification of circulatory miRNA detected in the plasma samples and is expressed
496 as log2 normalised Fragments Per Kilobase of transcript per Million mapped (FPKM)
497 reads. Differential expression was determined using the edgeR program
498 (Bioconductor software), and significant expression was defined as having a log fold
499 change greater than 2.0 and a false rate of discovery (FDR) adjusted P value $\leq$ 0.05.
500 Cluster analysis (Euclidean distance) informs the X and Y-axis dendrograms

501

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504 **Functional Analysis**

505 Causal inference analysis using Ingenuity Pathway analysis (IPA) software was
506 performed to identify the likely upstream regulators responsible for the changes in
507 mRNA and miRNA expression noted in the asthmatic subjects.

508 In total, 246 upstream gene regulators had a P value of overlap $\leq$ 0.05; indicating
509 that they have altered functional activity in the asthmatic subjects on the basis of
510 differential mRNA and miRNA expression. Of these regulators, seven had Z scores
511 greater than 2.0, thus enabling their activity to be predicted. Two upstream
512 regulators were predicted to have significantly increased activity in the asthmatic
513 subjects (P value of overlap $\leq$ 0.05; Z score $\geq$ 2.0), and five were predicted to have
514 significantly decreased activity asthmatic subjects (P value of overlap $\leq$ 0.05; Z score
515 $\leq$ -2.0) in the (**Table 5**).

516

517

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518 **Table 5: Upstream gene regulators with predicted significantly altered activity**
519 **in the asthmatic subjects (n = 4) compared to the control subjects (n = 5).**
520 Upstream regulators predicted to have significantly altered activity were defined as
521 having a P value of overlap $\leq$ 0.05 and a Z score greater than 2.0. Activated
522 upstream regulators are defined as having a Z score $\geq$ 2.0, and inhibited upstream
523 regulators are defined as having a Z score $\leq$ -2.0. Target molecules activated =
524 genes present in the RNA dataset that are activated by the upstream regulator;
525 target molecules inhibited = genes present in the RNA dataset that are inhibited by
526 the upstream regulator; target molecules affected = genes present in the RNA
527 dataset whose activity is known to be altered by the upstream regulator but there is
528 insufficient evidence to prove this is activation or inhibition.

529

Upstream Regulator	Molecule type	Activity state	Z score	P value of overlap	# Target molecules activated	# Target molecules inhibited	# Target molecules affected
Sirolimus	Chemical drug	Activated	2.75	0.0107	12	1	0
GFI1	Transcription regulator	Activated	2.00	0.0077	4	0	1
EIF4E	Transcription regulator	Inhibited	-2.00	0.0074	0	4	2
Mycophenolic acid	Chemical drug	Inhibited	-2.00	0.0211	0	4	0
Streptozocin	Chemical drug	Inhibited	-2.16	0.0492	0	5	1
SOX4	Transcription regulator	Inhibited	-2.24	0.0770	0	5	0
SYVN1	Transporter	Inhibited	-2.45	0.0069	0	6	0

530

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532 Of interest, with regards to atopic asthma pathology, was the predicted activated
533 state of GFI1, a transcription regulator induced by T cell activation and IL-4/STAT6
534 signalling. GFI1 is known to enhance Th2 expansion (71), and thus predicted
535 activation of this transcription regulator would suggest increased T cell activation and
536 subsequent expansion of the Th2 cell populations within the asthmatic cohort. This
537 notion is further supported by the prediction of significant inhibition of the upstream
538 regulator SOX4 in the asthmatic cohort. This transcription factor has been observed
539 to suppress Th2 differentiation (72), and thus its inhibition would allow expansion of
540 the Th2 populations within the asthmatic subjects. The predicted activated state of
541 GFI1 would also influence innate immune responses within the asthmatic cohort.
542 The transcription factor has been found to have a role in the development and
543 maintenance of type 2 innate lymphoid cells (73); a cell population that has been
544 found to be involved in allergic lung inflammation (74–76).

545 However, it should be noted causal inference analysis was performed on mRNA
546 detected in the blood, and thus the cellular origins of the gene expression observed
547 is unknown. Further study would be required to determine if GFI1 was indeed
548 activated and SOX4 was inhibited in the relevant body sites and or relevant *in vitro*
549 models of asthma pathology.

550

551 **Downstream Activity**

552 Causal inference analysis using IPA was also used to predict the downstream
553 consequences of the observed differential mRNA and miRNA expression within the
554 asthmatic subjects. The downstream effects of the differential expression were
555 primarily assessed by examination of the predicted canonical pathways and bio-
556 functions impacted.

557

558 **Canonical pathway analysis**

559 Fourteen canonical pathways were found to have significantly altered biological
560 activity ($P \leq 0.05$) within the asthmatic subjects (**Table 6**). In line with the findings of
561 the upstream analysis, a number of canonical pathways involved in T cell and B cell
562 activity, including signalling in rheumatoid arthritis, B cell development, and Nur77
563 signalling. It is interesting to note the canonical pathways involved in rheumatoid
564 arthritis and Type 1 diabetes were identified, as both diseases have been found to
565 display co-occurrence with asthma (77,78). It is tempting to speculate about the
566 existence of similar / shared underlying immune pathologies in the three diseases.

567

568

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569 **Table 6: Canonical signalling pathways predicted to have significantly altered**
570 **activity in the asthmatic subjects (n = 4) compared to the control subjects (n =**
571 **5).** Casual interference using Ingenuity Pathway Analysis (IPA) software was used to
572 predict downstream canonical signalling pathways likely to be affected by changes in
573 gene expression and regulation in the asthmatic subjects. Molecules with increased
574 gene expression are genes that had significantly increased numbers of mRNA reads
575 in the asthma plasma samples, and molecules with decreased gene expression are
576 genes that had significantly decreased numbers of mRNA reads in the asthma
577 plasma samples. Canonical pathways that are defined as being significantly altered
578 in the asthma subjects have a P value $\leq$ 0.05.

579

Canonical Pathway	P Value	Molecules with increased gene expression	Molecules with decreased gene expression
Altered T Cell and B Cell Signalling in Rheumatoid Arthritis	0.0053	SLAMF1,TLR1,HLA-DQA1,TNFRSF13C	HLA-DRB5
B Cell Development	0.0092	HLA-DQA1	IL7R, HLA-DRB5
Antigen Presentation Pathway	0.0116	HLA-DQA1, MR1	HLA-DRB5
Melatonin Degradation III	0.0124	MPO	-
TNFR1 Signalling	0.0241	-	TRADD, IKBKB, PAK4
Acute Myeloid Leukemia Signalling	0.0287	PML	CSF2RB, CEBPA, IDH3B
Tetrahydrobiopterin Biosynthesis I	0.0368	-	PTS
Hypusine Biosynthesis	0.0368	-	DOHH
Tetrahydrobiopterin Biosynthesis II	0.0368	-	PTS
Nur77 Signalling in T Lymphocytes	0.0369	HDAC9, HLA-DQA1	HLA-DRB5
Phagosome Maturation	0.0375	MPO, GOSR2	CTSL, CTSG, HLA-DRB5
Catecholamine Biosynthesis	0.0487	-	PNMT
Mitotic Roles of Polo-Like Kinase	0.0488	STAG2	ANAPC4, PPP2R5C
Type I Diabetes Mellitus Signalling	0.0496	HLA-DQA1	TRADD, IKBKB, HLA-DRB5

580

581

582 **Bio-function analysis**

583 With regards to biological functions likely to be impacted by changes in the observed
584 mRNA and miRNA expression patterns, a number of key immunological pathways
585 were predicted to have altered activity within the asthmatic cohort (Table 7).

586 Altered activity was defined as having a P value $\leq$ 0.05 and a Z score $\geq$ 2.0 or $\leq$ -2.0;
587 and in total 10 biological functions had significantly altered activity within the
588 asthmatic subjects (**Table 7**).

589

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590 **Table 7: Biological functions predicted to have significantly altered activity in**
591 **the asthmatic subjects (n = 4) compared to the control subjects (n = 5).** Casual
592 inference using Ingenuity Pathway Analysis (IPA) software was used to predict
593 biological functions likely to have altered activity in the asthmatic subjects. This was
594 determined through analysis of genes and miRNA that had altered expression in the
595 asthmatic subjects, to predict which biological functions would likely be altered.
596 Biological functions predicted to be significantly altered in the asthmatic subjects
597 were defined as having a P value $\leq$ 0.05 and a Z score greater than 2.0. Biological
598 functions with predicted increased activity were defined as having a Z score $\geq$ 2.0,
599 and biological functions with predicted decreased activity were defined as having a Z
600 score $\leq$ -2.0

601

Biological Functions	P Value	Activation State	Z score
Binding of endothelial cells	9.75E-03	Decreased	-2.123
Binding of leukocytes	1.73E-03	Decreased	-2.062
Cell transformation	1.32E-03	Decreased	-3.228
Differentiation of fibroblast cell lines	4.44E-03	Decreased	-2.184
Immune response of leukocytes	6.79E-04	Decreased	-2.031
Interaction of endothelial cells	3.55E-03	Decreased	-2.346
Killing of natural killer cells	5.44E-03	Decreased	-2.63
Proliferation of hepatocytes	6.53E-03	Increased	2.177
Tumorigenesis of tissue	4.94E-04	Increased	2.215
Viral infection	1.34E-02	Decreased	-2.099

602

603 Unsurprisingly, leukocyte activity was identified as being decreased in the asthmatic
604 cohort. However, at this level of analysis, the downstream effects on biological
605 function of the different classes of leukocytes was not determined, and thus further
606 study would be required to ascertain which leukocytes would likely have altered
607 activity in the asthmatic subjects as a consequence of the differential mRNA and
608 miRNA expression. Study of the specific leukocyte classes affected by asthma would
609 be crucial, as inhibition of the Th1 or Treg lymphocytes would likely enhance asthma
610 pathophysiology, whereas inhibition of the Th2 lymphocytes would likely alleviate
611 asthma pathophysiology.

612 It was also of interest to observe the predicted decrease in killing of natural killer
613 cells. This cell population has been previously identified as having a critical role in
614 immune defence against viruses and bacteria (79–82). In particular, viral infections
615 have been long characterised to exacerbate asthma (83–86), and asthmatics have
616 been observed to be deficient in type I IFN production (87–89), which likely
617 influences natural killer cell activity. Moreover, in a murine model, natural killer cell
618 activity was found to be decreased during a Th2 response (90). This suggests that in
619 asthmatic subjects, as a consequence of a Th2 biased immune system, there is
620 reduced natural killer cell activity, resulting in the known associations with asthma

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621 and respiratory infections. Moreover, this may also partially explain the changes in
622 the airway microbiome we see in asthmatic populations.

623

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Characterisation of the Blood Microbiota

Bacterial Relative Abundance

Our previous characterisation (38) of the bacterial RNA present in the plasma samples found that the majority of bacterial RNA belonged to the Proteobacteria phylum (Total relative abundance = 83.9%; Control mean = 90.0%; Asthma mean = 80.3%), the Actinobacteria phylum (Total relative abundance = 7.5%, Control mean = 6.0%, Asthma mean = 7.5%), and the Firmicutes phylum (Total relative abundance = 6.6%, Control mean = 3.0%, Asthma mean = 9.0%) (**Figure 6**). Please refer to (38) for a detailed appraisal of our experimental controls.

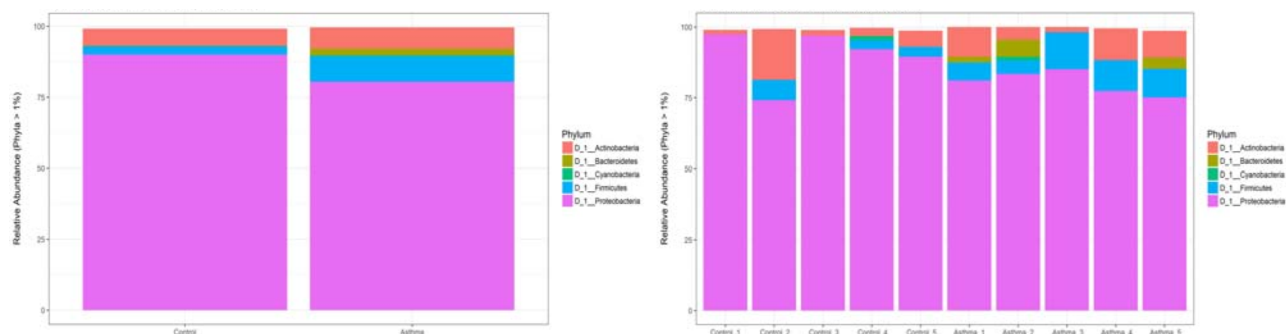

Figure 6: Microbial profile of the blood microbiome at the phylum level in asthmatic subjects (n = 5) and control subjects (n = 5). Composition of the blood microbiome was determined through sequencing of the bacterial V4 region of the 16S rRNA gene from bacterial DNA isolated from plasma samples from control subjects (n = 5) and asthmatic subjects (n = 5). The generated bacterial sequences were clustered (99% identity) in Operational Taxonomic Units (OTUs) to the Silva database and then assigned to bacterial taxonomic classes. **A** = microbial profile of the asthmatic subjects (n = 5) compared to the control subjects (n = 5). **B** = Microbial profiles of the individual plasma samples (n = 10)

In the asthmatic samples, 16S amplification and sequencing revealed a significant increase in Firmicutes (P value = 0.0148), associated with a concomitant decrease in Proteobacteria (P value = 0.0702) (**Figure 6**). To a lesser extent, members of the Bacteroidetes phylum were also detected in the blood samples, with increased levels of Bacteroidetes observed in the asthmatic subjects (Control mean relative abundance = 0.26%, range = 0.0 – 2.7%; Asthma mean relative abundance = 2.40%, range = 0 – 6.0%), although this was found to be non-significant increase (P value = 0.5258).

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656 **Lefse Analysis**

657

658 Analysis of the phyla relative abundances detected in the blood was achieved using
659 conventional statistical tests (unpaired *t* tests and Wilcox tests where appropriate)
660 and suggested significant differences in the blood microbiome between control and
661 asthma subjects. To test this, the linear discriminant analysis effect size (LefSe)
662 method was applied to the 16S rRNA relative abundance data to determine the
663 bacterial taxa most likely to explain the differences between the control and asthma
664 blood microbiomes. LefSe was also used to determine the biological consistency and
665 effect relevance of the observed differences in relative abundance.

666 In total, LefSe identified 8 bacterial taxa that showed statistically significant and
667 biologically consistent differences in the asthmatic subjects compared to the control
668 subjects (**Figure 7**). These findings were consistent with our previous analysis of the
669 bacterial populations using standard statistical tests (data not shown). Six of the
670 eight bacterial taxa displaying significant differences in relative abundance were
671 increased in the asthmatic subjects, whilst 2 bacterial taxa were decreased. At the
672 taxonomic class level, *Bacilli* were increased and *Bacteroidia* were decreased in the
673 asthmatic subjects, whilst at the genus level both *Kocuria* and *Stenotrophomonas*
674 were both increased in the asthmatic subjects.

675 The observed increases in Firmicutes were of particular interest as expansion of this
676 phylum has been associated with severe asthma (21). Furthermore, increased levels
677 of Firmicutes in the asthmatic subjects was predominately due to expansions of
678 *Staphylococcus and Streptococcus* genera, both of which have been associated with
679 the development of asthma during early childhood (91–94).

680 Additionally, our results were reflective of a previous study investigating the oral
681 microbiome, whereby Firmicutes, *Stenotrophomonas*, and *Lactobacillus* were found
682 to be increased in asthmatic subjects compared to the control subjects (95). This
683 suggests that bacterial nucleic acid detected in the blood may have originated from
684 the oral cavities, a theory that we consider in (38).

685

686

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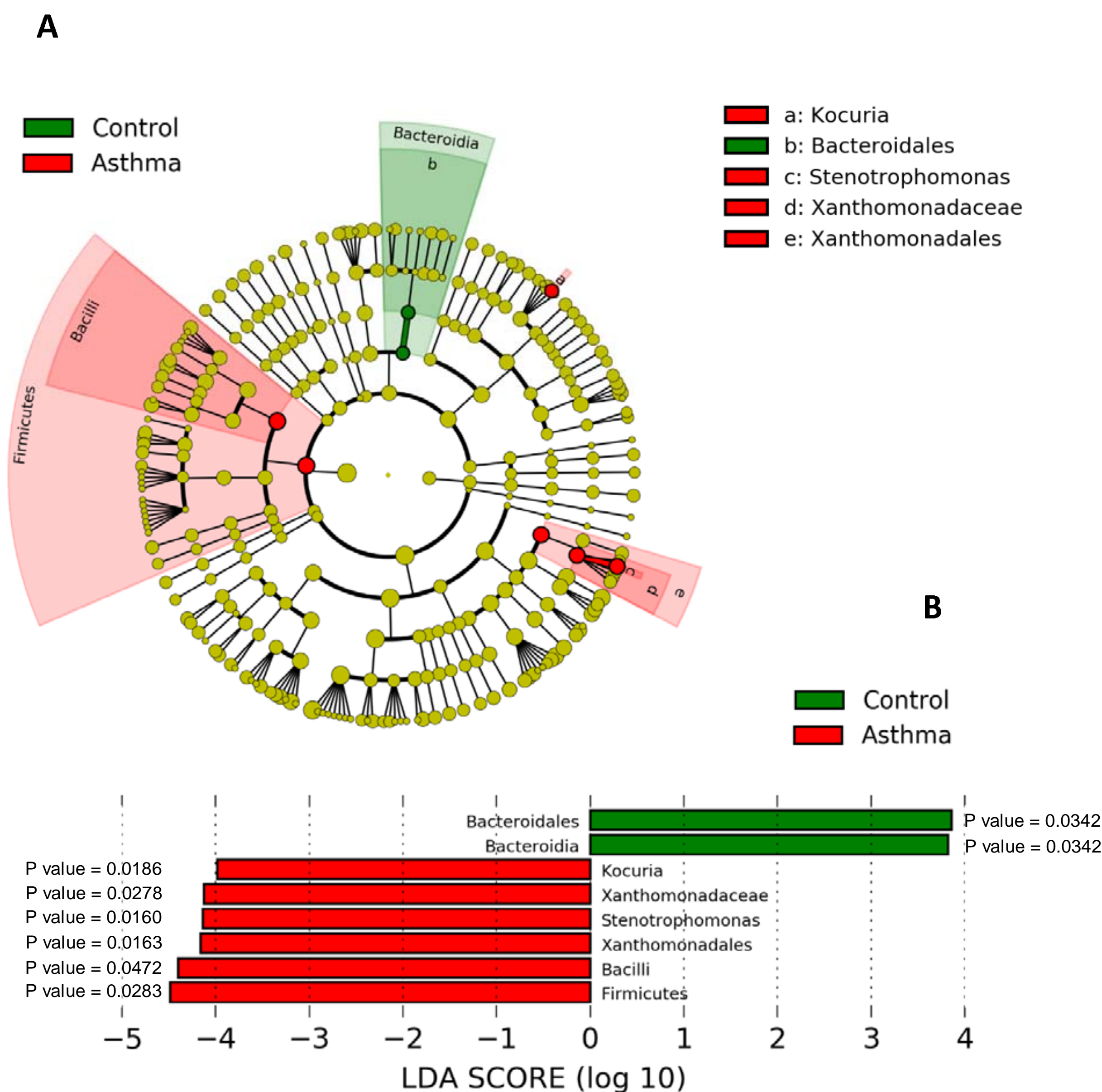
687 **Figure 7: Comparison of the healthy blood microbiome (n = 5) and the**
688 **asthmatic blood microbiome (n = 5) using LefSe**. Linear discriminant analysis
689 effect size (Lefse) analysis was performed on the bacterial taxa relative abundance
690 values to determine the presence of bacterial taxa with statistically significant
691 changes in abundance in the asthma blood microbiome compared to the control
692 blood microbiome. **A.** Taxonomic cladogram showing control enriched taxa (Green)
693 and asthma enriched taxa (Red). **B.** Effect size of the differential taxa. The control
694 enriched taxa are indicated with a positive LDA score, and the asthma enriched taxa
695 are indicated with a negative LDA score. The level of significance is indicated by the
696 P value shown for each taxa.

697



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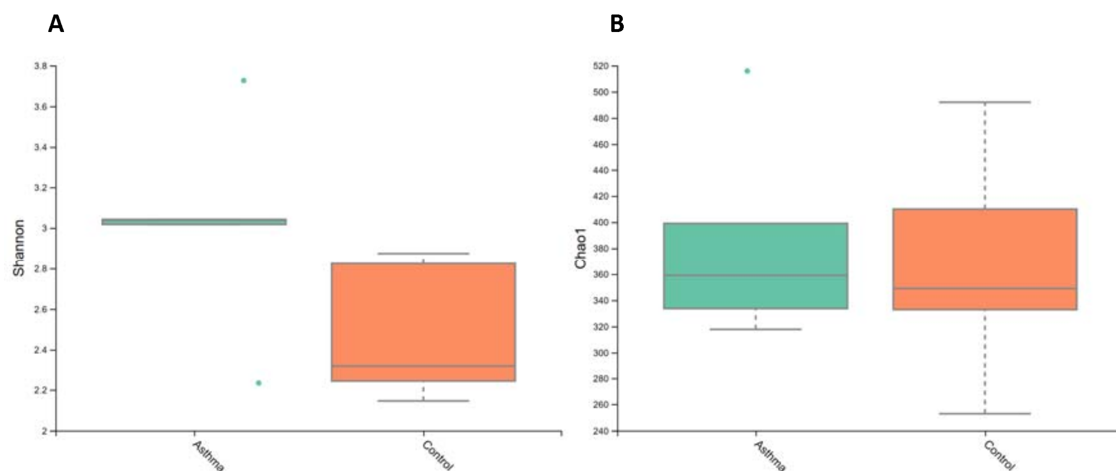
Bacterial Diversity

At the genus level, 81 bacterial genera were detected in the asthma plasma samples compared to 49 bacterial genera detected in the control plasma samples. Alpha and beta diversity of the bacterial populations present in the asthma and control groups was therefore assessed to determine whether there was significantly elevated bacterial diversity within the blood microbiome of the asthmatic subjects.

Alpha Diversity

Alpha diversity was determined by calculating the Chao1 index and Shannon index for each plasma sample. The control index scores were then compared to the asthma index scores to determine whether there were any significant differences between the two groups (Fig. 8).



Alpha Diversity	Control Mean (sd)	Asthma Mean (sd)	Fold Change	P value
Shannon	3.58 (0.44)	4.34 (0.68)	1.2130	0.0710
Chao1	378.90(58.12)	390.62 (62.44)	1.0309	0.7820

Figure 8: Comparison of alpha diversity present in the asthma blood microbiome compared to the control blood microbiome. Alpha diversity was measured using rarefied OTU tables generated from 16S rRNA sequencing data from plasma samples collected from asthma subjects (n = 5) and control subjects (n = 5). Shannon diversity index scores were generated from OTU tables in order to measure the richness of the plasma sample and evenness of bacterial taxa present in the sample. Chao1 index scores were measured to determine the predicted number of bacterial taxa present in the plasma samples by extrapolating out the number of rare organisms that may not have been detected due to under-sampling. **A** = Comparison of Shannon index scores generated from asthma plasma samples (n = 5) and control plasma samples (n = 5), **B =** Chao1 index scores generated from asthma plasma samples (n = 5) and control plasma samples (n = 5).

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725 Comparison between the asthma and control cohorts revealed that the asthmatic
726 subjects scored higher Chao1 and Shannon index scores than the control subjects,
727 thus suggesting that asthma is associated with increased bacterial diversity (**Figure**
728 **8**). This was particularly apparent for the Shannon diversity scores (P value =
729 0.0710) (**Figure 8**). Intriguingly, one of the asthma subjects, Asthma_3, displayed a
730 Shannon diversity score more similar to the controls than the other asthmatic
731 subjects. This subject developed asthma relatively late in childhood (age 12 years),
732 and so it is possible that the age of asthma onset may influence the level of microbial
733 diversity present in the blood. This is further supported by the high levels of alpha
734 diversity present in the blood of Asthma_5, an asthmatic subject who was diagnosed
735 with asthma early on in childhood (3 years).

736

737 **Beta Diversity**

738

739 Beta diversity was calculated to determine how similar the blood samples were to
740 one another with regards to bacterial diversity. This enabled not only comparison
741 between the asthma and control subjects, but also between the different members
742 within each group.

743 Beta diversity was determined by performing principal coordinate component (PCoA)
744 analysis using weighted UniFrac distances (**Figure 9**). PCoA analysis found that
745 beta diversity was principally a consequence of PCo1 variation (37.1%), and overall
746 the asthmatic subjects had higher PCo1 values with regards to beta diversity within
747 the blood microbiome compared to the control subjects.

748

749

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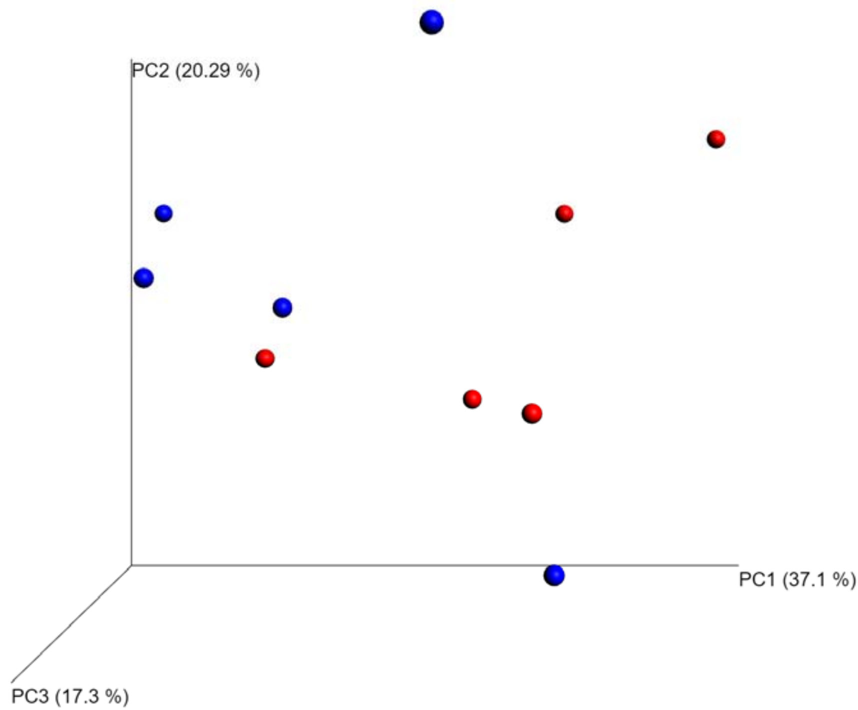
750



751

752

753 **Figure 9: Beta diversity of the blood microbiome from asthmatic subjects (n =**
754 **5) and control subjects (n = 5) using weighted UniFrac distance.** Principal
755 coordinate analysis (PCoA) was performed on OTU tables generated from 16S rRNA
756 sequencing data from plasma samples collected from asthma subjects (n = 5) and
757 control subjects (n = 5). Quantitative phylogenetic distances between each of the
758 samples was measured using a weighed UniFrac distance matrix, and the weighted
759 UniFrac distances were plotted as a PCoA graph to show beta diversity within
760 plasma samples from control subjects (n = 5; data plots = blue) and asthma subjects
761 (n = 5; data plots = red)

762

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Concluding Remarks

This study aimed to characterise a small yet specific population of HDM-sensitive adult asthma patients who had developed asthma during childhood. A range of molecular techniques was applied to characterise gene expression and regulation, inflammatory protein levels, and nucleic acid evidence of bacteria present in the blood. This was carried out in an effort to increase our understanding of this particular asthma phenotype, to begin to explore the molecular mechanisms responsible, and to identify any candidate biomarkers for further study.

At the protein level, the asthmatic subjects displayed increased inflammatory protein levels in the blood compared to the control subjects. This was particularly apparent for GM-CSF, IFN□, and TARC. The range of inflammatory protein levels within the asthmatic subjects was noticeably higher than the range observed for the control subjects. This was explained by the presence of two distinct clusters in the asthmatic cohort; cluster one was composed of subjects Asthma_2 and Asthma_4, and was characterised by high inflammatory protein levels; and cluster two, composed of Asthma_1, Asthma_3, and Asthma_5, and characterised by lower levels of inflammatory proteins. An association between the existence of other atopic complications, in particular evidence of atopic dermatitis, and IL-17A levels was unexpectedly observed.

Measurement of total IgE concentration within the blood revealed that IgE was detectable in half of the subjects under investigation (3 control subjects and 2 asthmatic subjects) and was significantly increased in the asthmatic subjects, when detected. The low detection rate of IgE was not unexpected given its short half-life (approximately two days) and low concentration levels within the blood (96). IgE was detected in asthma subjects belonging to the proposed cluster one, and this further supports the theory of asthmatic subjects forming sub-phenotypes on the basis of circulatory inflammation. In contrast to IgE, endotoxin levels were decreased in the asthmatic subjects (P value = 0.0650), and there appeared to be an inverse correlation between circulatory endotoxin levels and the reporting of additional atopic complications. This was a particularly interesting finding as exposure to endotoxin during early childhood has been previously found to be protective of the development of childhood asthma (97–100), and we were able to detect changes in endotoxin levels in our adult cohort.

Analysis of the diversity of RNA expression within the blood revealed that our asthmatic donors had more similar RNA profiles to one another than they did to the control subjects; this was particularly apparent in the miRNA analysis. When combined with our differential expression analyses, we identified specific mRNA and miRNA populations within the blood that were distinct between the healthy and disease states. Interestingly, asthma severity and the use of anti-inflammatory medication appeared to further influence RNA profiles although we note the

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808 limitations of our sample size, and acknowledge the need for a larger sample size to
809 explore this phenomenon fully. With regards to the unmet need for asthma
810 biomarkers, we identified various mRNAs in the circulation that were expressed in a
811 condition-specific manner, including HIST1H3C, PRAM1, RAB6B and CD93. Of
812 these, elevated levels of soluble CD93 have been previously reported in the serum
813 of asthmatics during acute asthma exacerbations (101) and in the serum of steroid-
814 naïve asthmatic patients (102).

815

816 Our microbial characterisation informed by 16S rRNA amplification and sequencing,
817 revealed increased levels of Firmicutes and decreased levels of Proteobacteria
818 within the blood of our asthmatic donors. This finding was accompanied by increased
819 bacterial diversity within the blood of asthmatic subjects, and the identification of
820 several additional bacterial taxa displaying significantly altered levels dependent on
821 disease state. The observed decrease in circulating Proteobacteria rRNA in the
822 asthmatic state is thought to be indicative of reduced Proteobacteria carriage within
823 the asthmatic subjects at a distant microbiome niche (e.g. the gut, airways and oral
824 cavity). This may explain the decreased levels of endotoxin (protein) detected in our
825 asthmatic subjects, given that endotoxin-producing gram-negative bacteria dominate
826 this phylum. Previous studies have associated childhood asthma and reduced
827 endotoxin exposure, and it is interesting to note that we detected this same
828 phenomenon in our adult asthma cohort, many years following childhood.
829 Furthermore, our asthma patients were found to have increased levels of
830 Bacteroidetes rRNA, and this appeared to be dependent on medication status with
831 those patients taking anti-inflammatory medications having lower levels of circulating
832 Bacteroidetes 16S rRNA than those who were not. As blood circulates the body and
833 functions as a medium that samples from virtually all body sites (103), it was not
834 possible to determine herein the microbial niche from which these signals originated.
835 That said, we hypothesise that changes in the blood are reflective of dysbiosis at
836 distant site(s) with well-characterised microbial communities (e.g. the gut, oral cavity
837 and skin), and have significant biomarker potential.

838

839 This study provides a valuable insight into the systemic changes evident in the HDM-
840 associated asthma, identifies a range of molecules that are present in the circulation
841 in a condition-specific manner (with clear biomarker potential), and highlights a range
842 of hypotheses for further study. Moreover, our data also provide an insight into the
843 level of heterogeneity observed both within the control and asthma samples
844 investigated, and will be of use for informing sample size calculations for future
845 studies.

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851 The National Institute for Health Research Health Protection Research Unit (NIHR
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855

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862

863 ## Competing Interests Statement

864

865 I declare that the authors have no competing interests as defined by Nature Research, or
866 other interests that might be perceived to influence the results and/or discussion reported in
867 this paper.

868

869 ## Author Contributions

870 DPT, MOL and TWG conceived the original study. DPT developed and refined the molecular
871 approach used. EW conducted the laboratory work. EW and DPT conducted the data
872 analysis. EW and DPT interpreted the original data. EW and DPT prepared the original
873 manuscript. EW, MOL, TWG and DPT reviewed and approved the manuscript.

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878 **References**

879 1. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma:
880 executive summary of the GINA Dissemination Committee Report. Allergy
881 [Internet]. 2004 May 1 [cited 2018 Aug 1];59(5):469–78. Available from:
882 http://doi.wiley.com/10.1111/j.1398-9995.2004.00526.x

883 2. Braman SS. The Global Burden of Asthma. Chest [Internet]. 2006 Jul 1 [cited
884 2018 Aug 1];130(1):4S–12S. Available from:
885 https://www.sciencedirect.com/science/article/pii/S0012369215329524

886 3. Asher I, Pearce N. Global burden of asthma among children. Int J Tuberc Lung
887 Dis [Internet]. 2014 Nov 1 [cited 2018 Aug 1];18(11):1269–78. Available from:
888 http://openurl.ingenta.com/content/xref?genre=article&issn=1027-
889 3719&volume=18&issue=11&spage=1269

890 4. Pawankar R. Allergic diseases and asthma: a global public health concern and
891 a call to action. World Allergy Organ J [Internet]. 2014 Dec 19 [cited 2018 Aug
892 1];7(1):1–3. Available from:
893 https://waojournal.biomedcentral.com/articles/10.1186/1939-4551-7-12

894 5. Pawankar R, Canonica G, Holgate S, Lockey R, Blaiss M. WAO White Book
895 on Allergy 2013 Update WAO White Book on Allergy WAO White Book on
896 Allergy [Internet]. [cited 2018 Aug 1]. Available from:
897 http://www.worldallergy.org/UserFiles/file/WhiteBook2-2013-v8.pdf

898 6. Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE. Distinguishing
899 severe asthma phenotypes: Role of age at onset and eosinophilic
900 inflammation. J Allergy Clin Immunol [Internet]. 2004 Jan 1 [cited 2018 Aug
901 1];113(1):101–8. Available from:
902 https://www.sciencedirect.com/science/article/pii/S0091674903024837

903 7. Holgate ST. Innate and adaptive immune responses in asthma. Nat Med
904 [Internet]. 2012 May 1 [cited 2018 Aug 1];18(5):673–83. Available from:
905 http://www.nature.com/articles/nm.2731

906 8. Lucini V, Ciracì R, Dugnani S, Pannacci M, Pisati F, Caronno A, et al.
907 Antibiotics counteract the worsening of airway remodelling induced by
908 infections in asthma. Int J Antimicrob Agents [Internet]. 2014 May 1 [cited 2018
909 Aug 1];43(5):442–50. Available from:
910 https://www.sciencedirect.com/science/article/pii/S0924857914000600

911 9. Postma DS, Bleecker ER, Amelung PJ, Holroyd KJ, Xu J, Panhuysen CIM, et
912 al. Genetic Susceptibility to Asthma — Bronchial Hyperresponsiveness
913 Coinherited with a Major Gene for Atopy. N Engl J Med [Internet]. 1995 Oct 5
914 [cited 2018 Aug 1];333(14):894–900. Available from:
915 http://www.nejm.org/doi/abs/10.1056/NEJM199510053331402

916 10. von Hertzen LC. Maternal stress and T-cell differentiation of the developing
917 immune system: Possible implications for the development of asthma and
918 atopy. J Allergy Clin Immunol [Internet]. 2002 Jun 1 [cited 2018 Aug
919 1];109(6):923–8. Available from:
920 https://www.sciencedirect.com/science/article/pii/S0091674902000040

921 11. Gilmour MI, Jaakkola MS, London SJ, Nel AE, Rogers CA. How exposure to

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922 environmental tobacco smoke, outdoor air pollutants, and increased pollen
923 burdens influences the incidence of asthma. Environ Health Perspect
924 [Internet]. 2006 Apr [cited 2018 Aug 1];114(4):627–33. Available from:
925 http://www.ncbi.nlm.nih.gov/pubmed/16581557

926 12. Vercelli D. Discovering susceptibility genes for asthma and allergy. Nat Rev
927 Immunol [Internet]. 2008 Mar 1 [cited 2018 Aug 1];8(3):169–82. Available from:
928 http://www.nature.com/articles/nri2257

929 13. Beasley R, Semprini A, Mitchell EA. Risk factors for asthma: is prevention
930 possible? Lancet [Internet]. 2015 Sep 12 [cited 2018 Aug 1];386(9998):1075–
931 85. Available from:
932 https://www.sciencedirect.com/science/article/pii/S0140673615001567

933 14. Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR, et al. A
934 genome-wide search for quantitative trait loci underlying asthma. Nature
935 [Internet]. 1996 Sep 19 [cited 2018 Aug 1];383(6597):247–50. Available from:
936 http://www.nature.com/doifinder/10.1038/383247a0

937 15. Anderson GP. Endotyping asthma: new insights into key pathogenic
938 mechanisms in a complex, heterogeneous disease. Lancet [Internet]. 2008
939 Sep 20 [cited 2018 Aug 1];372(9643):1107–19. Available from:
940 https://www.sciencedirect.com/science/article/pii/S014067360861452X

941 16. Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald JM, et
942 al. Global strategy for asthma management and prevention: GINA executive
943 summary. Eur Respir J [Internet]. 2008 Jan 1 [cited 2018 Aug 1];31(1):143–78.
944 Available from: http://www.ncbi.nlm.nih.gov/pubmed/18166595

945 17. Edwards MR, Bartlett NW, Hussell T, Openshaw P, Johnston SL. The
946 microbiology of asthma. Nat Rev Microbiol [Internet]. 2012 Jul 6 [cited 2018
947 Aug 1];10(7):459–71. Available from:
948 http://www.nature.com/articles/nrmicro2801

949 18. Wenzel SE. Asthma: defining of the persistent adult phenotypes. Lancet
950 [Internet]. 2006 Aug 26 [cited 2018 Aug 1];368(9537):804–13. Available from:
951 https://www.sciencedirect.com/science/article/pii/S0140673606692908

952 19. Lötvall J, Akdis CA, Bacharier LB, Bjermer L, Casale TB, Custovic A, et al.
953 Asthma endotypes: A new approach to classification of disease entities within
954 the asthma syndrome. J Allergy Clin Immunol [Internet]. 2011 Feb 1 [cited
955 2018 Aug 1];127(2):355–60. Available from:
956 https://www.sciencedirect.com/science/article/pii/S0091674910018580

957 20. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al.
958 Cluster Analysis and Clinical Asthma Phenotypes. Am J Respir Crit Care Med
959 [Internet]. 2008 Aug 20 [cited 2018 Aug 1];178(3):218–24. Available from:
960 http://www.atsjournals.org/doi/abs/10.1164/rccm.200711-1754OC

961 21. Zhang Q, Cox M, Liang Z, Brinkmann F, Cardenas PA, Duff R, et al. Airway
962 Microbiota in Severe Asthma and Relationship to Asthma Severity and
963 Phenotypes. Chalmers JD, editor. PLoS One [Internet]. 2016 Apr 14 [cited
964 2018 Aug 1];11(4):e0152724. Available from:
965 http://dx.plos.org/10.1371/journal.pone.0152724

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966 22. Dahl R. Systemic side effects of inhaled corticosteroids in patients with
967 asthma. Respir Med [Internet]. 2006 Aug 1 [cited 2018 Aug 1];100(8):1307–17.
968 Available from:
969 https://www.sciencedirect.com/science/article/pii/S095461110500510X

970 23. Guilbert TW, Morgan WJ, Zeiger RS, Mauger DT, Boehmer SJ, Szefler SJ, et
971 al. Long-Term Inhaled Corticosteroids in Preschool Children at High Risk for
972 Asthma. N Engl J Med [Internet]. 2006 May 11 [cited 2018 Aug
973 1];354(19):1985–97. Available from:
974 http://www.nejm.org/doi/abs/10.1056/NEJMoa051378

975 24. Cumming RG, Mitchell P, Leeder SR. Use of Inhaled Corticosteroids and the
976 Risk of Cataracts. N Engl J Med [Internet]. 1997 Jul 3 [cited 2018 Aug
977 1];337(1):8–14. Available from:
978 http://www.nejm.org/doi/abs/10.1056/NEJM199707033370102

979 25. Wang JJ, Rochtchina E, Tan AG, Cumming RG, Leeder SR, Mitchell P. Use of
980 Inhaled and Oral Corticosteroids and the Long-term Risk of Cataract.
981 Ophthalmology [Internet]. 2009 Apr 1 [cited 2018 Aug 1];116(4):652–7.
982 Available from:
983 https://www.sciencedirect.com/science/article/pii/S0161642008012542

984 26. Israel E, Banerjee TR, Fitzmaurice GM, Kotlov T V., LaHive K, LeBoff MS.
985 Effects of Inhaled Glucocorticoids on Bone Density in Premenopausal Women.
986 N Engl J Med [Internet]. 2001 Sep 27 [cited 2018 Aug 1];345(13):941–7.
987 Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa002304

988 27. Staa TP van, Staa TP van, Staa TP van, Leufkens HGM, Cooper C. The
989 Epidemiology of Corticosteroid-Induced Osteoporosis: a Meta-analysis.
990 Osteoporos Int [Internet]. 2002 Oct 1 [cited 2018 Aug 1];13(10):777–87.
991 Available from: http://link.springer.com/10.1007/s001980200108

992 28. Salpeter SR, Ormiston TM, Salpeter EE. Cardiovascular Effects of β-Agonists
993 in Patients With Asthma and COPD: A Meta-Analysis. Chest [Internet]. 2004
994 Jun 1 [cited 2018 Aug 1];125(6):2309–21. Available from:
995 https://www.sciencedirect.com/science/article/pii/S0012369216590102

996 29. Adcock IM, Caramori G, Chung KF. New targets for drug development in
997 asthma. Lancet [Internet]. 2008 Sep 20 [cited 2018 Aug 1];372(9643):1073–87.
998 Available from:
999 https://www.sciencedirect.com/science/article/pii/S014067360861449X

1000 30. Calderón MA, Linneberg A, Kleine-Tebbe J, De Blay F, Hernandez Fernandez
1001 de Rojas D, Virchow JC, et al. Respiratory allergy caused by house dust mites:
1002 What do we really know? J Allergy Clin Immunol [Internet]. 2015 Jul 1 [cited
1003 2018 Aug 1];136(1):38–48. Available from:
1004 https://www.sciencedirect.com/science/article/pii/S0091674914014821

1005 31. Kocabas CN, Civelek E, Sackesen C, Orhan F, Tuncer A, Adalioglu G, et al.
1006 Burden of rhinitis in children with asthma. Pediatr Pulmonol [Internet]. 2005
1007 Sep 1 [cited 2018 Aug 1];40(3):235–40. Available from:
1008 http://doi.wiley.com/10.1002/ppul.20247

1009 32. Kupczyk M, Kupryś I, Górski P, Kuna P. Aspirin intolerance and allergy to

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1010 house dust mites: important factors associated with development of severe
1011 asthma. Ann Allergy, Asthma Immunol [Internet]. 2004 Apr 1 [cited 2018 Aug
1012 1];92(4):453–8. Available from:
1013 http://linkinghub.elsevier.com/retrieve/pii/S1081120610617826

1014 33. Virchow JC, Backer V, Kuna P, Prieto L, Nolte H, Villesen HH, et al. Efficacy of
1015 a House Dust Mite Sublingual Allergen Immunotherapy Tablet in Adults With
1016 Allergic Asthma. JAMA [Internet]. 2016 Apr 26 [cited 2018 Aug
1017 1];315(16):1715. Available from:
1018 http://jama.jamanetwork.com/article.aspx?doi=10.1001/jama.2016.3964

1019 34. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential
1020 gene and transcript expression analysis of RNA-seq experiments with TopHat
1021 and Cufflinks. Nat Protoc [Internet]. 2012 Mar 1 [cited 2018 Aug 1];7(3):562–
1022 78. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22383036

1023 35. Rueda A, Barturen G, Lebrón R, Gómez-Martín C, Alganza Á, Oliver JL, et al.
1024 sRNAtoolbox: an integrated collection of small RNA research tools. Nucleic
1025 Acids Res [Internet]. 2015 Jul 1 [cited 2018 Oct 7];43(W1):W467-73. Available
1026 from: http://www.ncbi.nlm.nih.gov/pubmed/26019179

1027 36. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for
1028 differential expression analysis of digital gene expression data. Bioinformatics
1029 [Internet]. 2010 Jan 1 [cited 2018 Oct 7];26(1):139–40. Available from:
1030 https://academic.oup.com/bioinformatics/article-
1031 lookup/doi/10.1093/bioinformatics/btp616

1032 37. Krämer A, Green J, Pollard J, Tugendreich S. Causal analysis approaches in
1033 Ingenuity Pathway Analysis. Bioinformatics [Internet]. 2014 Feb 15 [cited 2018
1034 Aug 29];30(4):523–30. Available from:
1035 https://academic.oup.com/bioinformatics/article-
1036 lookup/doi/10.1093/bioinformatics/btt703

1037 38. Whittle E, Leonard MO, Harrison RD, Gant TW, Tonge DP. Multi-Method
1038 Characterisation of the Human Circulating Microbiome. bioRxiv [Internet]. 2018
1039 Jul 2 [cited 2018 Oct 10];359760. Available from:
1040 https://www.biorxiv.org/content/early/2018/07/02/359760

1041 39. Juniper EF, O'byrne PM, Guyatt G., Ferrie P., King D. Development and
1042 validation of a questionnaire to measure asthma control. Eur Respir J
1043 [Internet]. 1999 Oct 1 [cited 2018 Aug 1];14(4):902. Available from:
1044 http://erj.ersjournals.com/content/14/4/902

1045 40. Juniper EF, Gruffydd-Jones K, Ward S, Svensson K. Asthma Control
1046 Questionnaire in children: validation, measurement properties, interpretation.
1047 Eur Respir J [Internet]. 2010 Dec 7 [cited 2018 Aug 1];36(6):1410–6. Available
1048 from: http://www.ncbi.nlm.nih.gov/pubmed/20530041

1049 41. Liu J, Liu Y, Wang D, He M, Diao L, Liu Z, et al. AllerGAtlas 1.0: a human
1050 allergy-related genes database. Database [Internet]. 2018 Jan 1 [cited 2018
1051 Sep 24];2018. Available from:
1052 https://academic.oup.com/database/article/doi/10.1093/database/bay010/4904
1053 120

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1054 42. Kemper C, Chan AC, Green JM, Brett KA, Murphy KM, Atkinson JP. Activation
1055 of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1
1056 phenotype. Nature [Internet]. 2003 Jan 23 [cited 2018 Oct 1];421(6921):388–
1057 92. Available from: http://www.nature.com/doifinder/10.1038/nature01315

1058 43. Xu Y-Q, Gao Y-D, Yang J, Guo W. A defect of CD4+CD25+ regulatory T cells
1059 in inducing interleukin-10 production from CD4+ T cells under CD46
1060 costimulation in asthma patients. J Asthma [Internet]. 2010 May 9 [cited 2018
1061 Oct 1];47(4):367–73. Available from:
1062 http://www.ncbi.nlm.nih.gov/pubmed/20528588

1063 44. Cardone J, Le Friec G, Vantourout P, Roberts A, Fuchs A, Jackson I, et al.
1064 Complement regulator CD46 temporally regulates cytokine production by
1065 conventional and unconventional T cells. Nat Immunol [Internet]. 2010 Sep 8
1066 [cited 2018 Oct 2];11(9):862–71. Available from:
1067 http://www.nature.com/articles/ni.1917

1068 45. Le Friec G, Sheppard D, Whiteman P, Karsten CM, Shamoun SA-T, Laing A,
1069 et al. The CD46-Jagged1 interaction is critical for human TH1 immunity. Nat
1070 Immunol [Internet]. 2012 Dec 21 [cited 2018 Oct 2];13(12):1213–21. Available
1071 from: http://www.nature.com/articles/ni.2454

1072 46. Tsai Y-G, Niu D-M, Yang KD, Hung C-H, Yeh Y-J, Lee C-Y, et al. Functional
1073 defects of CD46-induced regulatory T cells to suppress airway inflammation in
1074 mite allergic asthma. Lab Investig [Internet]. 2012 Sep 2 [cited 2018 Oct
1075 1];92(9):1260–9. Available from:
1076 http://www.nature.com/articles/labinvest201286

1077 47. Simonetta F, Chiali A, Cordier C, Urrutia A, Girault I, Bloquet S, et al.
1078 Increased CD127 expression on activated FOXP3 1 CD4 1 regulatory T cells.
1079 [cited 2018 Oct 1]; Available from: www.eji-journal.eu

1080 48. Kelly EAB, Koziol-White CJ, Clay KJ, Liu LY, Bates ME, Bertics PJ, et al.
1081 Potential contribution of IL-7 to allergen-induced eosinophilic airway
1082 inflammation in asthma. J Immunol [Internet]. 2009 Feb 1 [cited 2018 Oct
1083 1];182(3):1404–10. Available from:
1084 http://www.ncbi.nlm.nih.gov/pubmed/19155487

1085 49. del Pozo V, Rojo M, Rubio ML, Cortegano I, Cárdaba B, Gallardo S, et al.
1086 Gene Therapy with Galectin-3 Inhibits Bronchial Obstruction and Inflammation
1087 in Antigen-challenged Rats through Interleukin-5 Gene Downregulation. Am J
1088 Respir Crit Care Med [Internet]. 2002 Sep 20 [cited 2018 Oct 1];166(5):732–7.
1089 Available from: http://www.atsjournals.org/doi/abs/10.1164/rccm.2111031

1090 50. Fermin Lee A, Chen H-Y, Wan L, Wu S-Y, Yu J-S, Huang AC, et al. Galectin-3
1091 Modulates Th17 Responses by Regulating Dendritic Cell Cytokines. Am J
1092 Pathol [Internet]. 2013 Oct 1 [cited 2018 Oct 1];183(4):1209–22. Available
1093 from: https://www.sciencedirect.com/science/article/pii/S0002944013004732

1094 51. Zhang R, Brennan ML, Shen Z, Macpherson JC, Schmitt D, Molenda C, et al.
1095 Myeloperoxidase Functions as a Major Enzymatic Catalyst for Initiation of Lipid
1096 Peroxidation at Sites of Inflammation Downloaded from [Internet]. Vol. 1. JBC
1097 Papers in Press; 2002 [cited 2018 Oct 1]. Available from:
1098 http://www.jbc.org/content/early/2002/09/30/jbc.M209124200.full.pdf

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1099 52. Carraway R, Cochrane DE, Lansman JB, Leeman SE, Paterson BM, Welch
1100 HJ. Neurotensin stimulates exocytotic histamine secretion from rat mast cells
1101 and elevates plasma histamine levels. J Physiol [Internet]. 1982 Feb 1 [cited
1102 2018 Oct 1];323(1):403–14. Available from:
1103 http://doi.wiley.com/10.1113/jphysiol.1982.sp014080

1104 53. Singh LK, Pang X, Alexacos N, Letourneau R, Theoharides TC. Acute
1105 Immobilization Stress Triggers Skin Mast Cell Degranulation via Corticotropin
1106 Releasing Hormone, Neurotensin, and Substance P: A Link to Neurogenic
1107 Skin Disorders. Brain Behav Immun [Internet]. 1999 Sep 1 [cited 2018 Oct
1108 1];13(3):225–39. Available from:
1109 https://www.sciencedirect.com/science/article/pii/S0889159198905414

1110 54. Hatzelmann A, Schudt C. Anti-Inflammatory and Immunomodulatory Potential
1111 of the Novel PDE4 Inhibitor Roflumilast in Vitro [Internet]. 2001 [cited 2018 Oct
1112 2]. Available from: http://jpet.aspetjournals.org

1113 55. Tang H-F, Song Y-H, Chen J-C, Chen J-Q, Wang P. Upregulation of
1114 Phosphodiesterase-4 in the Lung of Allergic Rats. Am J Respir Crit Care Med
1115 [Internet]. 2005 Apr 15 [cited 2018 Oct 2];171(8):823–8. Available from:
1116 http://www.ncbi.nlm.nih.gov/pubmed/15665325

1117 56. Sun J, Deng Y, Wu X, Tang H, Deng J, Chen J, et al. Inhibition of
1118 phosphodiesterase activity, airway inflammation and hyperresponsiveness by
1119 PDE4 inhibitor and glucocorticoid in a murine model of allergic asthma. Life Sci
1120 [Internet]. 2006 Oct 26 [cited 2018 Oct 2];79(22):2077–85. Available from:
1121 https://www.sciencedirect.com/science/article/pii/S0024320506005236

1122 57. Wyllie DH, Kiss-Toth E, Visintin A, Smith SC, Boussouf S, Segal DM, et al.
1123 Evidence for an accessory protein function for Toll-like receptor 1 in anti-
1124 bacterial responses. J Immunol [Internet]. 2000 Dec 15 [cited 2018 Oct
1125 2];165(12):7125–32. Available from:
1126 http://www.ncbi.nlm.nih.gov/pubmed/11120843

1127 58. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting
1128 edge: role of Toll-like receptor 1 in mediating immune response to microbial
1129 lipoproteins. J Immunol [Internet]. 2002 Jul 1 [cited 2018 Oct 2];169(1):10–4.
1130 Available from: http://www.ncbi.nlm.nih.gov/pubmed/12077222

1131 59. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor
1132 triggering of a vitamin D-mediated human antimicrobial response. Science
1133 [Internet]. 2006 Mar 24 [cited 2018 Oct 2];311(5768):1770–3. Available from:
1134 http://www.ncbi.nlm.nih.gov/pubmed/16497887

1135 60. Wittke A, Weaver V, Mahon BD, August A, Cantorna MT. Vitamin D receptor-
1136 deficient mice fail to develop experimental allergic asthma. J Immunol
1137 [Internet]. 2004 Sep 1 [cited 2018 Oct 1];173(5):3432–6. Available from:
1138 http://www.ncbi.nlm.nih.gov/pubmed/15322208

1139 61. Hartmann B, Heine G, Babina M, Steinmeyer A, Zügel U, Radbruch A, et al.
1140 Targeting the vitamin D receptor inhibits the B cell-dependent allergic immune
1141 response. Allergy [Internet]. 2011 Apr 1 [cited 2018 Oct 1];66(4):540–8.
1142 Available from: http://doi.wiley.com/10.1111/j.1398-9995.2010.02513.x

bioRxiv preprint doi: https://doi.org/10.1101/446427; this version posted October 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1143 62. Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory
1144 cells suppress naive and memory T cell proliferation and can be expanded in
1145 vitro without loss of function. J Exp Med [Internet]. 2001 Jun 4 [cited 2018 Oct
1146 7];193(11):1295–302. Available from:
1147 http://www.ncbi.nlm.nih.gov/pubmed/11390436

1148 63. Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T
1149 regulatory cells. Immunol Rev [Internet]. 2001 Aug 1 [cited 2018 Oct
1150 7];182(1):68–79. Available from: http://doi.wiley.com/10.1034/j.1600-
1151 065X.2001.1820105.x

1152 64. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T Cells and
1153 Immune Tolerance. Cell [Internet]. 2008 May 30 [cited 2018 Oct
1154 7];133(5):775–87. Available from:
1155 https://www.sciencedirect.com/science/article/pii/S0092867408006247

1156 65. Shevach EM. Mechanisms of Foxp3+ T Regulatory Cell-Mediated
1157 Suppression. Immunity [Internet]. 2009 May 22 [cited 2018 Oct 7];30(5):636–
1158 45. Available from:
1159 https://www.sciencedirect.com/science/article/pii/S1074761309001976

1160 66. Muñoz-Culla M, Irizar H, Castillo-Triviño T, Sáenz-Cuesta M, Sepúlveda L,
1161 Lopetegi I, et al. Blood miRNA expression pattern is a possible risk marker for
1162 natalizumab-associated progressive multifocal leukoencephalopathy in
1163 multiple sclerosis patients. Mult Scler J [Internet]. 2014 Dec 22 [cited 2018 Aug
1164 31];20(14):1851–9. Available from:
1165 http://journals.sagepub.com/doi/10.1177/1352458514534513

1166 67. Nielsen S, Åkerström T, Rinnov A, Yfanti C, Scheele C, Pedersen BK, et al.
1167 The miRNA Plasma Signature in Response to Acute Aerobic Exercise and
1168 Endurance Training. Eckel J, editor. PLoS One [Internet]. 2014 Feb 19 [cited
1169 2018 Aug 31];9(2):e87308. Available from:
1170 http://dx.plos.org/10.1371/journal.pone.0087308

1171 68. Pirola CJ, Fernández Gianotti T, Castaño GO, Mallardi P, San Martino J, Mora
1172 Gonzalez Lopez Ledesma M, et al. Circulating microRNA signature in non-
1173 alcoholic fatty liver disease: from serum non-coding RNAs to liver histology
1174 and disease pathogenesis. Gut [Internet]. 2015 May [cited 2018 Aug
1175 31];64(5):800–12. Available from:
1176 http://www.ncbi.nlm.nih.gov/pubmed/24973316

1177 69. Panganiban RP, Wang Y, Howrylak J, Chinchilli VM, Craig TJ, August A, et al.
1178 Circulating microRNAs as biomarkers in patients with allergic rhinitis and
1179 asthma. J Allergy Clin Immunol [Internet]. 2016 May [cited 2018 Aug
1180 29];137(5):1423–32. Available from:
1181 http://linkinghub.elsevier.com/retrieve/pii/S0091674916003006

1182 70. Tonge DP, Gant TW. What is normal? Next generation sequencing-driven
1183 analysis of the human circulating miRNAOme. BMC Mol Biol [Internet]. 2016
1184 Dec 9 [cited 2018 Oct 10];17(1):4. Available from:
1185 http://www.biomedcentral.com/1471-2199/17/4

1186 71. Zhu J, Jankovic D, Grinberg A, Guo L, Paul WE. Gfi-1 plays an important role
1187 in IL-2-mediated Th2 cell expansion [Internet]. [cited 2018 Aug 29]. Available

bioRxiv preprint doi: https://doi.org/10.1101/446427; this version posted October 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1188 from: www.pnas.orgcgidoi10.1073pnas.0608981103

1189 72. Kuwahara M, Yamashita M, Shinoda K, Tofukuji S, Onodera A, Shinnakasu R,
1190 et al. The transcription factor Sox4 is a downstream target of signaling by the
1191 cytokine TGF-β and suppresses TH2 differentiation. Nat Immunol [Internet].
1192 2012 Aug 1 [cited 2018 Aug 29];13(8):778–86. Available from:
1193 http://www.nature.com/articles/ni.2362

1194 73. Spooner CJ, Lesch J, Yan D, Khan AA, Abbas A, Ramirez-Carrozzi V, et al.
1195 Specification of type 2 innate lymphocytes by the transcriptional determinant
1196 Gfi1. Nat Immunol [Internet]. 2013 Dec 20 [cited 2018 Aug 30];14(12):1229–
1197 36. Available from: http://www.nature.com/articles/ni.2743

1198 74. Chang Y-J, Kim HY, Albacker LA, Baumgarth N, McKenzie ANJ, Smith DE, et
1199 al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity
1200 independently of adaptive immunity. Nat Immunol [Internet]. 2011 Jul 29 [cited
1201 2018 Aug 30];12(7):631–8. Available from:
1202 http://www.nature.com/articles/ni.2045

1203 75. Halim TYF, Krauß RH, Sun AC, Takei F. Lung Natural Helper Cells Are a
1204 Critical Source of Th2 Cell-Type Cytokines in Protease Allergen-Induced
1205 Airway Inflammation. Immunity [Internet]. 2012 Mar 23 [cited 2018 Aug
1206 30];36(3):451–63. Available from:
1207 https://www.sciencedirect.com/science/article/pii/S1074761312000854

1208 76. Zhu J. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2)
1209 development and regulation of interleukin-4 (IL-4) and IL-13 production.
1210 Cytokine [Internet]. 2015 Sep 1 [cited 2018 Aug 30];75(1):14–24. Available
1211 from: https://www.sciencedirect.com/science/article/pii/S1043466615001908

1212 77. Shen T-C, Lin C-L, Wei C-C, Tu C-Y, Li Y-F. The risk of asthma in rheumatoid
1213 arthritis: a population-based cohort study. QJM [Internet]. 2014 Jun 1 [cited
1214 2018 Aug 30];107(6):435–42. Available from:
1215 https://academic.oup.com/qjmed/article-lookup/doi/10.1093/qjmed/hcu008

1216 78. Seiskari T, Viskari H, Kondrashova A, Haapala A-M, Ilonen J, Knip M, et al.
1217 Co-occurrence of allergic sensitization and type 1 diabetes. Ann Med
1218 [Internet]. 2010 Jul 13 [cited 2018 Aug 30];42(5):352–9. Available from:
1219 http://www.ncbi.nlm.nih.gov/pubmed/20465355

1220 79. Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. NATURAL
1221 KILLER CELLS IN ANTIVIRAL DEFENSE: Function and Regulation by Innate
1222 Cytokines. Annu Rev Immunol [Internet]. 1999 Apr 28 [cited 2018 Sep
1223 2];17(1):189–220. Available from:
1224 http://www.annualreviews.org/doi/10.1146/annurev.immunol.17.1.189

1225 80. French AR, Yokoyama WM. Natural killer cells and viral infections. Curr Opin
1226 Immunol [Internet]. 2003 Feb 1 [cited 2018 Sep 2];15(1):45–51. Available from:
1227 https://www.sciencedirect.com/science/article/pii/S095279150200002X

1228 81. Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the
1229 response to microorganisms. Nat Rev Microbiol [Internet]. 2007 Jun 8 [cited
1230 2018 Sep 2];5(6):405–17. Available from:
1231 http://www.nature.com/articles/nrmicro1657

bioRxiv preprint doi: https://doi.org/10.1101/446427; this version posted October 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1232 82. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural
1233 killer cells. 2008 [cited 2018 Sep 2]; Available from:
1234 http://www.nature.com/natureimmunology

1235 83. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of
1236 asthma in adults. BMJ [Internet]. 1993 Oct 16 [cited 2018 Sep
1237 2];307(6910):982–6. Available from:
1238 http://www.ncbi.nlm.nih.gov/pubmed/8241910

1239 84. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, et
1240 al. Community study of role of viral infections in exacerbations of asthma in 9-
1241 11 year old children. BMJ [Internet]. 1995 May 13 [cited 2018 Sep
1242 2];310(6989):1225–9. Available from:
1243 http://www.ncbi.nlm.nih.gov/pubmed/7767192

1244 85. Murray CS, Poletti G, Kebadze T, Morris J, Woodcock A, Johnston SL, et al.
1245 Study of modifiable risk factors for asthma exacerbations: virus infection and
1246 allergen exposure increase the risk of asthma hospital admissions in children.
1247 Thorax [Internet]. 2006 May 1 [cited 2018 Sep 2];61(5):376–82. Available from:
1248 http://www.ncbi.nlm.nih.gov/pubmed/16384881

1249 86. Busse WW, Lemanske RF, Gern JE. Role of viral respiratory infections in
1250 asthma and asthma exacerbations. Lancet [Internet]. 2010 Sep 4 [cited 2018
1251 Sep 2];376(9743):826–34. Available from:
1252 https://www.sciencedirect.com/science/article/pii/S0140673610613803

1253 87. Papadopoulos NG, Stanciu LA, Papi A, Holgate ST, Johnston SL. A defective
1254 type 1 response to rhinovirus in atopic asthma. Thorax [Internet]. 2002 Apr 1
1255 [cited 2018 Sep 2];57(4):328–32. Available from:
1256 http://www.ncbi.nlm.nih.gov/pubmed/11923551

1257 88. Wark PAB, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca
1258 V, et al. Asthmatic bronchial epithelial cells have a deficient innate immune
1259 response to infection with rhinovirus. J Exp Med [Internet]. 2005 Mar 21 [cited
1260 2018 Sep 2];201(6):937–47. Available from:
1261 http://www.ncbi.nlm.nih.gov/pubmed/15781584

1262 89. Sykes A, Edwards MR, Macintyre J, del Rosario A, Bakhsoliani E, Trujillo-
1263 Torralbo M-B, et al. Rhinovirus 16–induced IFN-α and IFN-β are deficient in
1264 bronchoalveolar lavage cells in asthmatic patients. J Allergy Clin Immunol
1265 [Internet]. 2012 Jun 1 [cited 2018 Sep 2];129(6):1506–1514.e6. Available from:
1266 https://www.sciencedirect.com/science/article/pii/S0091674912006173

1267 90. Openshaw PJ, Hussell T. Intracellular IFN-gamma expression in natural killer
1268 cells precedes lung CD8+ T cell recruitment during respiratory syncytial virus
1269 infection. J Gen Virol [Internet]. 1998 Nov 1 [cited 2018 Sep 2];79(11):2593–
1270 601. Available from:
1271 http://jgv.microbiologyresearch.org/content/journal/jgv/10.1099/0022-1317-79-
1272 11-2593

1273 91. Sears MR, Greene JM, Willan AR, Taylor DR, Flannery EM, Cowan JO, et al.
1274 Long-term relation between breastfeeding and development of atopy and
1275 asthma in children and young adults: a longitudinal study. Lancet [Internet].
1276 2002 Sep 21 [cited 2018 Sep 7];360(9337):901–7. Available from:

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1277 https://www.sciencedirect.com/science/article/pii/S0140673602110257

1278 92. Davis MF, Peng RD, McCormack MC, Matsui EC. Staphylococcus aureus
1279 colonization is associated with wheeze and asthma among US children and
1280 young adults. J Allergy Clin Immunol [Internet]. 2015 Mar 1 [cited 2018 Sep
1281 7];135(3):811–3.e5. Available from:
1282 http://www.ncbi.nlm.nih.gov/pubmed/25533526

1283 93. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bønnelykke
1284 K, et al. Childhood Asthma after Bacterial Colonization of the Airway in
1285 Neonates. N Engl J Med [Internet]. 2007 Oct 11 [cited 2018 Sep
1286 7];357(15):1487–95. Available from:
1287 http://www.nejm.org/doi/abs/10.1056/NEJMoa052632

1288 94. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The Infant
1289 Nasopharyngeal Microbiome Impacts Severity of Lower Respiratory Infection
1290 and Risk of Asthma Development. Cell Host Microbe [Internet]. 2015 May 13
1291 [cited 2018 Sep 7];17(5):704–15. Available from:
1292 https://www.sciencedirect.com/science/article/pii/S1931312815001250

1293 95. Park H, Shin JW, Park S-G, Kim W. Microbial Communities in the Upper
1294 Respiratory Tract of Patients with Asthma and Chronic Obstructive Pulmonary
1295 Disease. Chu HW, editor. PLoS One [Internet]. 2014 Oct 16 [cited 2018 Aug
1296 31];9(10):e109710. Available from:
1297 http://dx.plos.org/10.1371/journal.pone.0109710

1298 96. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils.
1299 J Allergy Clin Immunol [Internet]. 2010 Feb 1 [cited 2018 Sep 3];125(2):S73–
1300 80. Available from:
1301 https://www.sciencedirect.com/science/article/pii/S0091674909017345

1302 97. Gereda J, Leung D, Thatayatikom A, Streib J, Price M, Klinnert M, et al.
1303 Relation between house-dust endotoxin exposure, type 1 T-cell development,
1304 and allergen sensitisation in infants at high risk of asthma. Lancet [Internet].
1305 2000 May 13 [cited 2018 Sep 3];355(9216):1680–3. Available from:
1306 https://www.sciencedirect.com/science/article/pii/S014067360002239X

1307 98. Douwes J, van Strien R, Doekes G, Smit J, Kerkhof M, Gerritsen J, et al. Does
1308 early indoor microbial exposure reduce the risk of asthma? The Prevention
1309 and Incidence of Asthma and Mite Allergy birth cohort study. J Allergy Clin
1310 Immunol [Internet]. 2006 May 1 [cited 2018 Sep 3];117(5):1067–73. Available
1311 from: https://www.sciencedirect.com/science/article/pii/S0091674906003113

1312 99. Gehring U, Strikwold M, Schram-Bijkerk D, Weinmayr G, Genuneit J, Nagel G,
1313 et al. Asthma and allergic symptoms in relation to house dust endotoxin:
1314 Phase Two of the International Study on Asthma and Allergies in Childhood
1315 (ISAAC II). Clin Exp Allergy [Internet]. 2008 Dec 1 [cited 2018 Sep
1316 3];38(12):1911–20. Available from: http://doi.wiley.com/10.1111/j.1365-
1317 2222.2008.03087.x

1318 100. Tischer C, Gehring U, Chen C-M, Kerkhof M, Koppelman G, Sausenthaler S,
1319 et al. Respiratory health in children, and indoor exposure to (1,3)-β-D-glucan,
1320 EPS mould components and endotoxin. Eur Respir J [Internet]. 2011 May 3
1321 [cited 2018 Sep 3];37(5):1050–9. Available from:

bioRxiv preprint doi: https://doi.org/10.1101/446427; this version posted October 19, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1322 http://www.ncbi.nlm.nih.gov/pubmed/20817706

1323 101. Sigari N, Jalili A, Mahdawi L, Ghaderi E, Shilan M. Soluble CD93 as a Novel
1324 Biomarker in Asthma Exacerbation. Allergy Asthma Immunol Res [Internet].
1325 2016 Sep 1 [cited 2018 Sep 2];8(5):461. Available from:
1326 https://synapse.koreamed.org/DOIx.php?id=10.4168/aair.2016.8.5.461

1327 102. Park HJ, Han H, Lee SC, Son YW, Sim DW, Park KH, et al. Soluble CD93 in
1328 Serum as a Marker of Allergic Inflammation. Yonsei Med J [Internet]. 2017
1329 May 1 [cited 2018 Sep 2];58(3):598. Available from:
1330 https://synapse.koreamed.org/DOIx.php?id=10.3349/ymj.2017.58.3.598

1331 103. Kowarsky M, Camunas-Soler J, Kertesz M, De Vlaminck I, Koh W, Pan W, et
1332 al. Numerous uncharacterized and highly divergent microbes which colonize
1333 humans are revealed by circulating cell-free DNA. Proc Natl Acad Sci U S A
1334 [Internet]. 2017 Sep 5 [cited 2018 Oct 10];114(36):9623–8. Available from:
1335 http://www.ncbi.nlm.nih.gov/pubmed/28830999

1336

1337