Circulating MicroRNAs and Treatment Response in Childhood Asthma

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Online Data Supplement

Supplemental Methods

Participant selection

The CAMP trial (ClinicalTrials.gov Identifier: NCT00000575) was a 4-6 year long multicenter randomized controlled trial measuring the effects of budesonide, nedocromil, and a placebo on asthma outcomes in 1041 participants 5-12 years of age. The protocol and primary outcomes have been published previously (1, 2). Entry criteria included asthma symptoms and/or medication use for ⩾6 months in the previous year and airway responsiveness with PC20 ⩽ 12.5 mg/ml. Exclusion criteria included FEV1 < 65% of predicted when off β-agonists for > 4 hours, other active pulmonary disease, and the inability to perform acceptable spirometry, or to complete the study protocol requirements. During the study period, patients were randomized to inhaled budesonide 200 μg twice daily, nedocromil 8mg twice daily, or placebo twice daily (2). During the trial follow-up, visits occurred at 2 and 4 months post-randomization, then every 4 months thereafter. Visits consisted of height and weight measurements, spirometry, and questionnaires to assess asthma symptoms, health care utilization, and prednisone courses, all standardized across the eight CAMP clinical centers (1). CAMP procedures were approved by Institutional Review Boards at each of the clinical centers and for the CAMP Data Coordinating Center.

For the current study, we selected 462 participants from the CAMP subjects (188 to the budesonide treatment group and 274 to the placebo group) based on availability of serum (3). These participants provided the serum samples at baseline and were followed up for a mean 4.3 years. Lung function, including pre-bronchodilator FEV1 as a percent of predicted (FEV1%), was measured at randomization and each follow-up visit as below (4, 5). Our primary outcome was defined as the difference of FEV1% between the 48-month follow-up visit and randomization.

Pulmonary Function

Pulmonary function was determined by spirometry performed on a Collins Stead-Wells dry-seal Survey III spirometer both before and after bronchodilator (two 90-µg actuations of a pressurized metered-dose inhaler); measures of percent of predicted forced expiratory volume in the first second (FEV_1) and forced vital capacity (FVC), and FEV_1/FVC as percent were obtained. At least three acceptable maneuvers meeting American Thoracic Society (ATS) standards were required, with at least two reproducible (FEV1 and FVC within 5% of best) maneuvers required for each test. Equations for FEV_1% for age, sex and height were race-corrected according to Coultas and coworkers for Hispanics and Knudson and coworkers for all other ethnic groups (4, 5). Airway hyper responsiveness was defined as the concentration of methacholine that caused a decrease of 20% from baseline FEV_1 (PC20- FEV_1) (6).

Small RNA-Seq and profiling

Total RNA was isolated from serum samples by the Qiagen miRNeasy Serum/Plasma extraction kit and QIAcube automation. All samples were quantified using the Nanodrop spectrophotometer prior to plating. Small RNA-seq libraries were prepared using the Norgen Biotek Small RNA Library Prep Kit and then sequenced on the Illumina NextSeq 500 platform at 51bp single end reads. ExceRpt was employed to assess the read quality and annotate miRNAs (7).

The read count was log transformed and normalized by quantile normalization (R package: preprocessCore) (8). Raw read counts less than five were filtered out and miRNAs with coverage less than 80% of all subjects were removed. Coverage refers to the number of subjects in which a specific miRNA was detected at >=5 counts. The data has been submitted to GEO (GSE134897).

Statistical analysis

Linear regression was used to investigate the association between miRNAs and the change of FEV_1% over the course of the 4-year clinical trial. Age, sex, race, total eosinophil count and atopic dermatitis diagnosed by doctor were considered *a priori* as confounders in the regression model. The association between miRNAs and the change of FEV_1% was studied through both univariate and multivariate linear regression model stratified by treatment groups first, and then the multivariate interaction between miRNA and treatment groups were considered. The false discovery rate (FDR) was calculated using the R package 'fdrtool' (9). As we planned *a priori* to perform functional validation, the FDR threshold was set at 0.10.

Permutation test was employed on significant miRNAs for both the main effects analysis and the interaction effects analysis for additional statistical validation. We shuffled the sample outcomes (change in FEV_1%) and permuted 10,000 times for each miRNA. A permuted p-value for each miRNA was calculated under the multivariate linear regression model.

Functional Validation

The effect of miRNA on ICS response was studied in vitro using lung epithelial cells. The prevailing paradigm in ICS therapy is that glucocorticoid receptors directly prevent inflammatory gene transcription via repression of key inflammatory factors such as NF-κB and activator protein 1 (10, 11). A number of studies have used A549 cells to investigate the mechanisms of the anti-inflammatory effect of corticosteroids in asthma, mostly through reduction of NF-κB signaling (11-13). Immuno-histochemical analyses of biopsies from asthmatic patients under budesonide treatment also revealed that airway epithelial cells exhibit expression of the GILZ (glucocorticoid-induced leucine zipper) protein, a protein known to be induced by corticosteroid and represses NF-κB transcription (13, 14). We therefore estimated the function of miRNAs on glucocorticoid

induced trans-repression through an established reporter cell line A549/NF-κB-luc (15, 16). These cells are responsive to IL-1β stimulation, which is reduced by dexamethasone (Dex), a glucocorticoid receptor agonist, indicating that these cells exhibit glucocorticoid-mediated tethered transrepression of NF-κB. A549/NF-κB-luc reporter cells were transfected with either 25 nM of scramble control (AllStars Negative Control siRNA, Qiagen) or the indicated miRNA mimics (Qiagen) using RNAiMax (Life Technology) according to the manufacturer's protocol. After 48 hours post-transfection, the cells were stimulated with 5 ng/mL IL1β ± 10 nM dexamethasone. Luciferase assays were performed after 18 h treatment (see Figure2A). The luciferase activity in scramble control cells with 5 ng/mL IL-1β treatment was normalized to 1. The luciferase activities of cells treated with IL-1β + Dex and transfected with respective mimics were calculated and compared to that of cells treated with IL-1β + Dex and transfected with scramble control. Each treatment condition has 4 replicates.

Prediction

We built a multivariate logistic regression model to predict the ICS response in the treatment group. In order to decrease the intrinsic difference of FEV_1%, we predicted the highest vs. lowest quartiles of response. For this, 86 ICS treated subjects, including 43 highly responsive subjects and 43 poorly responsive subjects, were selected. The normalized miRNA counts with the potential confounders, including age, sex, race, total eosinophil count and atopic dermatitis, were set as input variables and the ICS responses were set as outcome (high lung function Y=1, low lung function Y=0).

Supplemental References

1. The Childhood Asthma Management Program (CAMP): design, rationale, and methods. Childhood Asthma Management Program Research Group. *Control Clin Trials* 1999; 20: 91-120.

2. Childhood Asthma Management Program Research G, Szefler S, Weiss S, Tonascia J, Adkinson NF, Bender B, Cherniack R, Donithan M, Kelly HW, Reisman J, Shapiro GG, Sternberg AL, Strunk R, Taggart V, Van Natta M, Wise R, Wu M, Zeiger R. Long-term effects of budesonide or nedocromil in children with asthma. *N Engl J Med* 2000; 343: 1054-1063.

3. Covar RA, Fuhlbrigge AL, Williams P, Kelly HW, the Childhood Asthma Management Program Research G. The Childhood Asthma Management Program (CAMP): Contributions to the Understanding of Therapy and the Natural History of Childhood Asthma. *Curr Respir Care Rep* 2012; 1: 243-250.

4. Coultas DB, Howard CA, Skipper BJ, Samet JM. Spirometric prediction equations for Hispanic children and adults in New Mexico. *Am Rev Respir Dis* 1988; 138: 1386-1392.

5. Knudson RJ, Lebowitz MD, Holberg CJ, Burrows B. Changes in the normal maximal expiratory flow-volume curve with growth and aging. *Am Rev Respir Dis* 1983; 127: 725-734.

6. Program CAM. Childhood Asthma Management Program for Methacholine Challenge Testing, Version 3.0. Springfield, VA: National Technical Information Service; 1994.

7. Rozowsky J, Kitchen RR, Park JJ, Galeev TR, Diao J, Warrell J, Thistlethwaite W, Subramanian SL, Milosavljevic A, Gerstein M. exceRpt: A Comprehensive Analytic Platform for Extracellular RNA Profiling. *Cell Syst* 2019; 8: 352-357 e353.

8. Bolstad B. preprocessCore: a collection of pre-processing functions. R package, version 1.44. 0. 2018.

9. Strimmer K. fdrtool: a versatile R package for estimating local and tail area-based false discovery rates. *Bioinformatics* 2008; 24: 1461-1462.

10. Barnes PJ. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *Br J Pharmacol* 2006; 148: 245-254.

11. Kelly MM, King EM, Rider CF, Gwozd C, Holden NS, Eddleston J, Zuraw B, Leigh R, O'Byrne PM, Newton R. Corticosteroid-induced gene expression in allergen-challenged asthmatic subjects taking inhaled budesonide. *Br J Pharmacol* 2012; 165: 1737-1747.

12. Kaur M, Chivers JE, Giembycz MA, Newton R. Long-acting beta2-adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription in human airway epithelial and smooth muscle cells. *Mol Pharmacol* 2008; 73: 203-214.

13. Kelly MM, O'Connor TM, Leigh R, Otis J, Gwozd C, Gauvreau GM, Gauldie J, O'Byrne PM. Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. *J Allergy Clin Immunol* 2010; 125: 349-356 e313.

14. Eddleston J, Herschbach J, Wagelie-Steffen AL, Christiansen SC, Zuraw BL. The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. *J Allergy Clin Immunol* 2007; 119: 115-122.

15. Jiang X, Dahlin A, Weiss ST, Tantisira K, Lu Q. A high-throughput chemical screen identifies novel inhibitors and enhancers of anti-inflammatory functions of the glucocorticoid receptor. *Sci Rep* 2017; 7: 7405.

16. McGeachie MJ, Clemmer GL, Hayete B, Xing H, Runge K, Wu AC, Jiang X, Lu Q, Church B, Khalil I, Tantisira K, Weiss S. Systems biology and in vitro validation identifies family with sequence similarity 129 member A (FAM129A) as an asthma steroid response modulator. *J Allergy Clin Immunol* 2018; 142: 1479-1488 e1412.

17. Jardim MJ, Dailey L, Silbajoris R, Diaz-Sanchez D. Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene. *Am J Respir Cell Mol Biol* 2012; 47: 536-542.

18. Panganiban RP, Wang Y, Howrylak J, Chinchilli VM, Craig TJ, August A, Ishmael FT. Circulating microRNAs as biomarkers in patients with allergic rhinitis and asthma. *J Allergy Clin Immunol* 2016; 137: 1423-1432.

Supplemental Tables

Table E1. Summary of small RNA-Seq data QC.

	Average Sample	Total Sample
Input	19,122,488	9,408,264,238
Successfully clipped	15,997,444	7,870,742,240
Reads used for alignment	14,009,818	6,892,830,326
Reads mapped to genome	4,063,818	1,999,398,334
miRNA	1,230,913	605,609,029
miRNA precursor	5,212	2,564,286
tRNA	1,174,200	577,706,581
piRNA	60,680	29,854,400
rRNA	867,903	427,008,085
circular RNA	32	15,541
gencode	866,724	426,428,460

Table E2. Permutation test for the significant miRNAs in budesonide (ICS) treatment group.

miRNA	Regression P value	Permutation Test (N=10000) P value
hsa-miR-155-5p	0.002	0.0023
hsa-miR-532-5p	0.002	0.0018
hsa-miR-4433b-5p	0.003	0.0028
hsa-miR-345-5p	0.003	0.0031
hsa-miR-652-3p	0.02	0.0213
hsa-miR-126-3p	0.02	0.0218
hsa-miR-335-5p	0.03	0.0278

Table E3. Permutation test for the significant miRNAs by the treatment interaction analysis.

miRNA	Regression P value	Permutation Test (N=10000) P value
hsa-miR-345-5p	0.0003	0.0004
hsa-miR-155-5p	0.002	0.0014
hsa-miR-652-3p	0.002	0.0015
hsa-miR-4433b-5p	0.003	0.0023
hsa-miR-335-5p	0.005	0.0044
hsa-let-7e-5p	0.006	0.0066
hsa-miR-532-5p	0.006	0.0071
hsa-miR-500a-3p	0.007	0.0062
hsa-miR-15b-5p	0.01	0.0122
hsa-miR-1180-3p	0.02	0.0197
hsa-miR-186-5p	0.02	0.0276
hsa-miR-126-3p	0.03	0.036
hsa-miR-424-3p	0.03	0.0331
hsa-miR-425-3p	0.03	0.0325
hsa-miR-502-3p	0.04	0.0392

Supplemental Figures

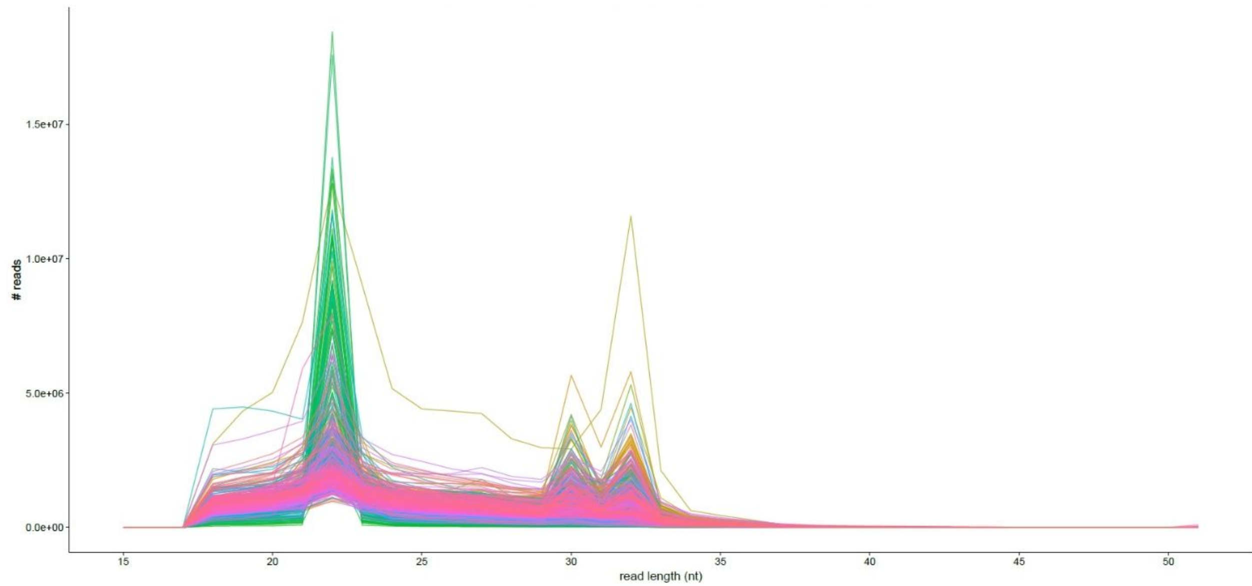

Figure E1. Read length distribution of sequenced samples based on number of raw reads. The raw reads from small RNA-Seq are 51bp in length with a significant peak at 22 bp according to the distribution of read length, the size expected for miRNAs.

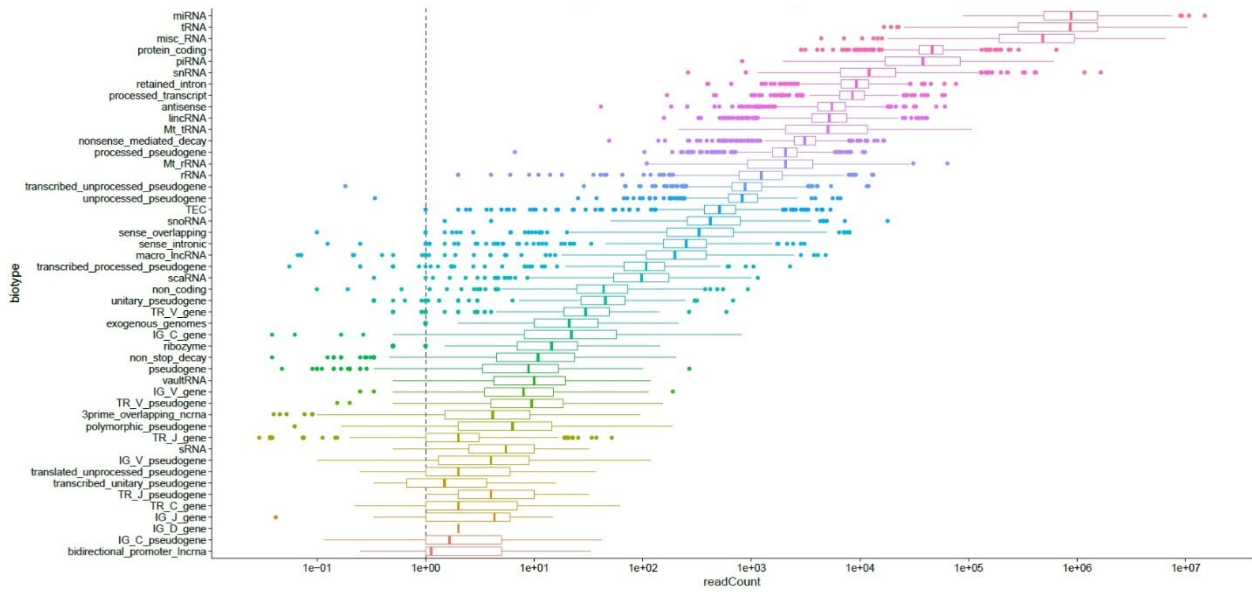

Figure E2. Overall biotype distribution within the NGS library based on raw read counts. Among all biotypes identified, miRNA reads make up the largest proportion of read count.

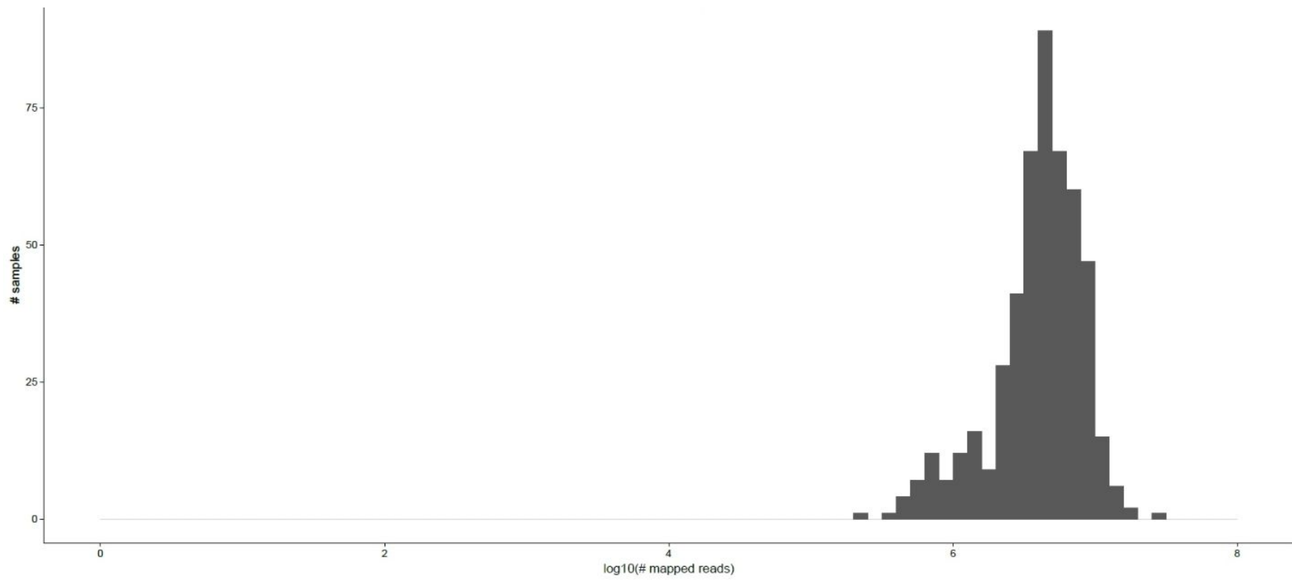

Figure E3. Histogram of library size (mapped reads). Our small RNA-Seq samples have a large abundance of mapped reads. About 90% of samples contain more than one million (10^6) reads.

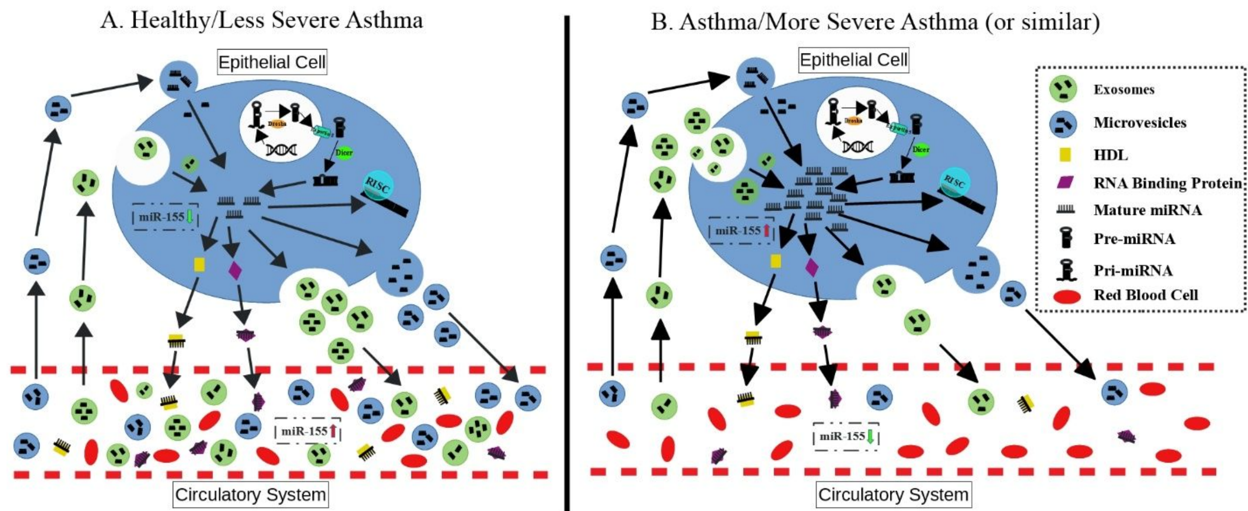

Figure E4. **Model of proposed miR-155 actions**. Intracellular and extracellular miR-155 level between healthy/less severe asthma and asthma/more severe asthma group. MiR-155 decreases intracellularly in epithelial cells in healthy/less severe asthma individuals, while it increases in asthma/more severe asthma individuals (17). In contrast, circulating miRNAs appear to be anti-correlated with intracellular miRNAs with regard to asthma or asthma severity (18), with healthy subjects having more miR-155 in the circulatory system and less in epithelial cells, while asthmatics have less miR-155 in the circulatory system and more in epithelial cells. Given that our CAMP samples are extracellular (serum), higher miR-155 concentrations (equating to improved ICS response) likely represent a less severe (more healthy) form of asthma.