

Journal Pre-proof

The Potential of microRNAs as Non-invasive Biomarkers in Acute Pediatric Asthma

Aleksandra Trifunovic, DO, Alan Dombkowski, PhD, Daniela Cukovic, Prashant Mahajan, MD MPH MBA



PII: S0091-6749(20)30121-4

DOI: <https://doi.org/10.1016/j.jaci.2020.01.032>

Reference: YMAI 14399

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 7 January 2019

Revised Date: 29 December 2019

Accepted Date: 8 January 2020

Please cite this article as: Trifunovic A, Dombkowski A, Cukovic D, Mahajan P, The Potential of microRNAs as Non-invasive Biomarkers in Acute Pediatric Asthma, *Journal of Allergy and Clinical Immunology* (2020), doi: <https://doi.org/10.1016/j.jaci.2020.01.032>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology.

TITLE PAGE

The Potential of microRNAs as Non-invasive Biomarkers in Acute Pediatric Asthma

Aleksandra Trifunovic DO,^a Alan Dombkowski PhD,^b Daniela Cukovic,^b Prashant Mahajan MD MPH MBA^c

From ^aDenver, CO, ^bWayne State University, ^cUniversity of Michigan.
Email: pmahajan@med.umich.edu

This project was supported by the Children's Hospital of Michigan and the Children's Research Center of Michigan (grant no. 10-CHM-0485). The content is solely the responsibility of the authors and does not necessarily represent the official views of the sponsor.

Disclosure of potential conflict of interest: The authors declare that they have no conflict of interest and nothing to disclose.

Keywords: microRNA expression, pediatric asthma, pediatric emergency medicine, miR-582-5p

Word Count: 928

Corresponding Author:
Prashant Mahajan, MD, MPH, MBA
Department of Emergency Medicine
University of Michigan
1540 E. Hospital Drive, CW 2-737
Ann Arbor, MI 48109-4260
(734) 763-9309
(734)-232-2666 (Fax)
pmahajan@med.umich.edu

Capsule Summary:

Our study demonstrated that it's feasible to obtain quality microRNAs from nasal epithelium in ED setting. We found that miR-582-5p is overexpressed in severe asthmatics suggesting future potential of this microRNA as a clinical biomarker.

The Potential of microRNAs as Non-invasive Biomarkers in Acute Pediatric Asthma

To the Editor:

Asthma affects nearly 6 million children in the United States. Approximately 50% of children with asthma experience an exacerbation annually, 16.7% require an emergency department (ED) visit and nearly 4.7% are hospitalized yearly, making asthma the third leading cause of pediatric inpatient admissions.¹

Asthma is a multifactorial disease often triggered by environmental factors in genetically predisposed individuals. Since the ED is frequently the first, if not the only, access to health care for many children with acute asthma, it provides a unique, yet largely unexplored, clinical setting to investigate novel molecular biomarkers for asthma severity and treatment response. MicroRNAs are a class of short, highly stable endogenous non-coding RNAs involved in regulating multiple cellular processes, either by preventing translation and/or inducing transcript degradation.² Past research has primarily focused on identifying changes in microRNA expression associated with airway inflammation in acute asthma,³ however, there is a paucity of pediatric data as these studies were primarily in adults, set in subacute care settings and utilized invasive methods of sample collection either via bronchoscopy or blood samples.

We sought to evaluate microRNA expression patterns in a pediatric ED population with varying asthma severity by using tissue obtained via nasal swabs. Nasal and bronchial cells have identical morphology and similar responses to cytokines in vivo and in vitro;⁴ therefore, nasal epithelia could potentially constitute an accessible surrogate for studying lower airway inflammation. The main objectives of our study were to demonstrate the feasibility of obtaining adequate amounts of high quality microRNAs via nasal swabs and to compare microRNA expression patterns in pediatric patients with varying severity of asthma.

We conducted a prospective, observational study on children ≤ 18 years of age presenting to an inner city, freestanding pediatric ED. This study was approved by the Institutional Review Board and patients were enrolled after obtaining informed consent. We enrolled patients in one of the following four cohorts: severe asthma exacerbation (SA); moderate asthma exacerbation (MA); history of asthma with no exacerbation (NE) and patients with no history of asthma or atopy (NC) (Table I). We defined acute asthma as wheezing or respiratory distress in a patient with prior diagnosis of asthma or

reactive airway disease. Asthma severity was determined using the Pediatric Asthma Severity Score (PASS), a standardized asthma assessment tool. We excluded patients with history of heart disease, lung disease other than asthma, and those who refused informed consent.

We enrolled a total of 50 participants and collected nasal swabs from each patient. RNA was successfully extracted and tested for quality from 48 specimens (Supplementary Methods in Online Repository). MicroRNA expression microarrays were performed for ~10 patient samples having the best RNA quality in each group (N=39 total, Supplementary Methods and Table E1 in Online Repository). Initial statistical analysis revealed expression values of some microRNAs were dependent on RNA quality. Therefore, for subsequent statistical analysis we excluded 11 samples with an RNA quality score below 7 (Supplementary Methods). The remaining 28 microarrays included 8 samples from each of the control (NC) and severe (SA) cohorts, and 6 samples from both the moderate (MA) and no exacerbation (NE) cohorts.

Using stringent statistical analysis where the probability of a false positive was restricted to 1%, comparison of severe and control groups revealed that microRNA miR-582-5p is significantly induced in the severe cohort (~24-fold, Figure I). The expression level of miR-582-5p in the moderate and no exacerbation groups is intermediate to the control and severe groups (Figure E1 in Online Repository). Interestingly, miR-582-5p has been found to be up-regulated in sputum of subjects exposed to ozone, altering their respiratory immune response.⁵ We investigated if the increase of miR-582-5p expression might be due to alterations in cell populations. Using a set of microRNAs characteristic for lymphocytes, RBCs, immune cells, and epithelial cells, we compared expression between the severe and control groups. No significant differences were observed (Figure E2 and Supplementary Methods in Online Repository).

Our study demonstrated that it is feasible to obtain adequate quantities of high quality microRNAs using a non-invasive approach and conduct complex genomic studies on pediatric asthma in the ED. While the use of blood and/or bronchial epithelial cells has been a preferred method of obtaining “ideal” tissue sample for biomarker studies in the past, this approach is not always practical, especially in the pediatric population. Here, we show that nasal epithelium can be used as a bronchial epithelium surrogate obtained via a substantially less invasive method compared to endobronchial lavage.

There are several study limitations. First is the small cohort size. While prior research revealed that significant differences in gene expression are detectable in small sample sizes ($N \leq 30$)⁶, our findings warrant further validation.

However, our statistical analysis showed that significant changes in microRNA

expression could be detected with a low false positive rate in nasal epithelia, even in a limited cohort. We found that high- quality RNAs are important to obtain robust results. Further improvements in the collection protocol may provide improved consistency in RNA quality. Second, the majority of enrolled patients were African-American, reflecting the demographics of our ED population. A multicenter study with a diverse population would be an optimal setting to validate our results.

In conclusion, it is feasible to obtain high quality microRNAs from nasal epithelium in pediatric patients presenting to the ED. In this proof-of-principle study, we demonstrated that miR-582-5p is robustly overexpressed in nasal epithelia of children with severe, acute asthma. Subsequent studies and validation are warranted to further investigate the relationship between miR-582-5p and asthma severity, and explore the potential of this microRNA as a clinical biomarker and to help guide targeted therapies.

We thank the patients and their families for their kind cooperation. We also thank Elizabeth Duffy for her research and editorial support.

Aleksandra Trifunovic DO,^a

Alan Dombkowski PhD,^b

Daniela Cukovic,^b

Prashant Mahajan MD MPH MBA^c

References

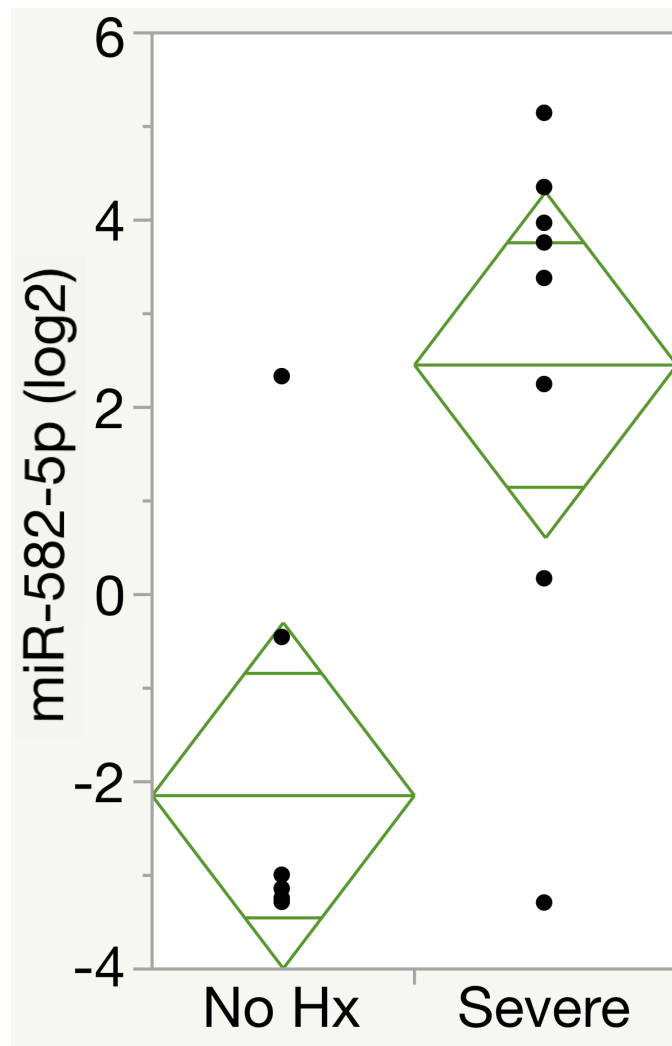
1. Zahran HS, Bailey CM, Damon SA, Garbe PL, Breysse PN. *Vital Signs: Asthma in Children — United States, 2001–2016*. MMWR Morb Mortal Wkly Rep 2018; 67:149–155. DOI: <http://dx.doi.org/10.15585/mmwr.mm6705e1>
2. Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297.
3. Lu TX, Munitz A, Rothenberg ME (2009). MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. J Immunol 182(8): 4994-5002.
4. McDougall CM, Blaylock MG, Douglas JG, Brooker RJ, Helms PJ and Walsh GM (2008) Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. Am J Respir Cell Mol Biol 39:560–568
5. Fry RC, Rager JE, Bauer R, Sebastian E, Peden DB, Jaspersl, et al. “Air toxics and epigenetic effects: ozone altered microRNAs in the sputum of human subjects”. *American Journal of Physiology—Lung Cellular and Molecular Physiology*. 2014;306(12):L1129–L1137
6. McGeachie MJ, Davis JS, Kho AT, Dahlin A, Sordillo JE, Sun M, et al. (2017). “Asthma remission: Predicting future airways responsiveness using an miRNA network.” J Allergy Clinical Immunol, 140(2):598-600.e8.

Figure Captions

Figure I. MicroRNA miR-582-5p is overexpressed in nasal epithelia of pediatric patients with severe asthma. Data points representing normalized expression of individual subjects are shown (N=8 for each group). Diamonds indicate confidence intervals, with mean values and overlap marks shown as horizontal bars. Statistical significance is indicated by vertical separation of the overlap marks for the two groups.

Table I. Patient Demographics

General	Total (N=39)	No History of Asthma (NC) (N=10)	History of Asthma with no Exacerbation (NA) (N=10)	Moderate Asthma Exacerbation (MA) (N=10)	Severe Asthma Exacerbation (SA) (N=9)
Sex					
Male	23	7	5	8	3
Female	16	3	5	2	6
Age					
<1 year	1	1	0	0	0
1-10 years	22	4	5	5	8
>10 years	16	5	5	5	1
Ethnicity					
White	4	3	0	1	0
African-American	35	7	10	9	9
Hispanic	0	0	0	0	0
Family History					
Asthma	29	3	8	9	9
Eczema	23	3	6	7	7
Patient History of Eczema	19	0	6	6	7

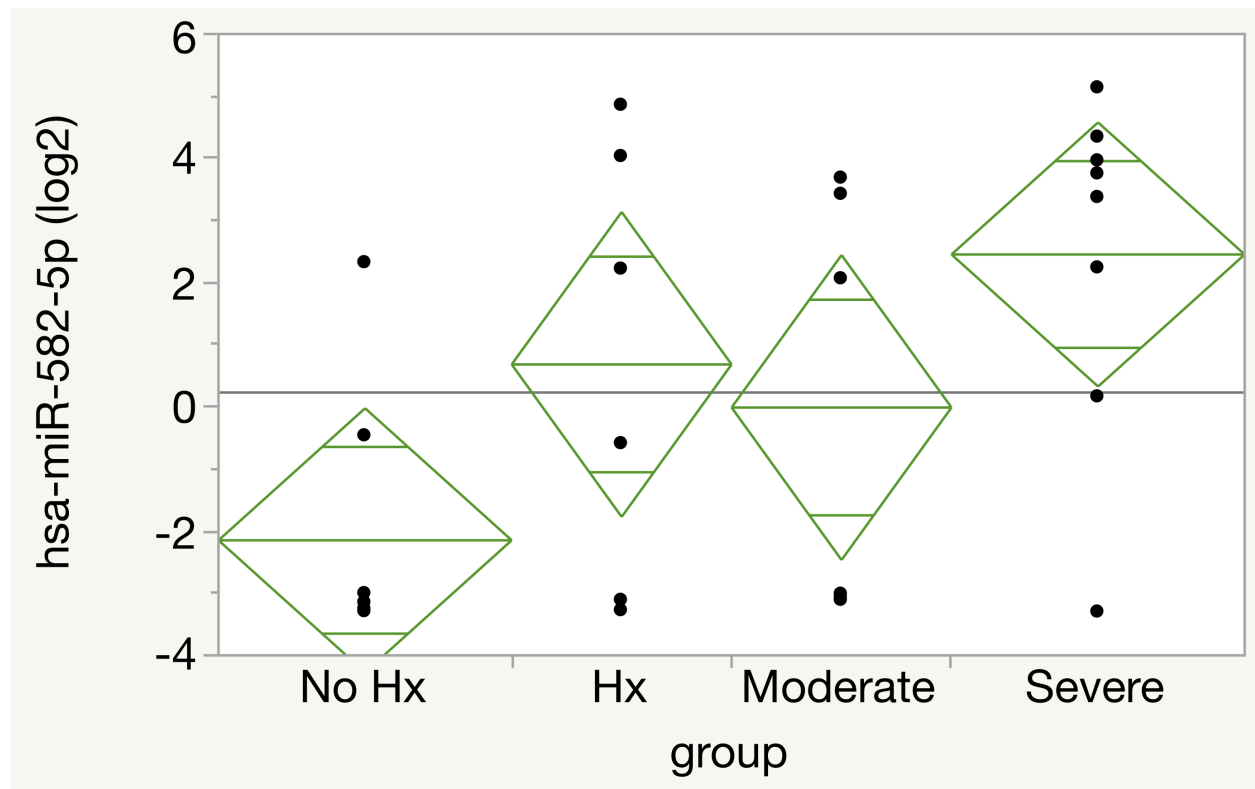


Supplemental Figure Legends

Supplementary Figure E1. MicroRNA miR-582-5p is in nasal epithelia is associated with asthma severity in pediatric patients. Data points representing normalized expression of individual subjects are shown (N=8 for each of No Hx and Severe, N=6 for Hx and Moderate). Diamonds indicate confidence intervals, with mean values and overlap marks shown as horizontal bars. Statistical significance between two groups is indicated by vertical separation of overlap marks.

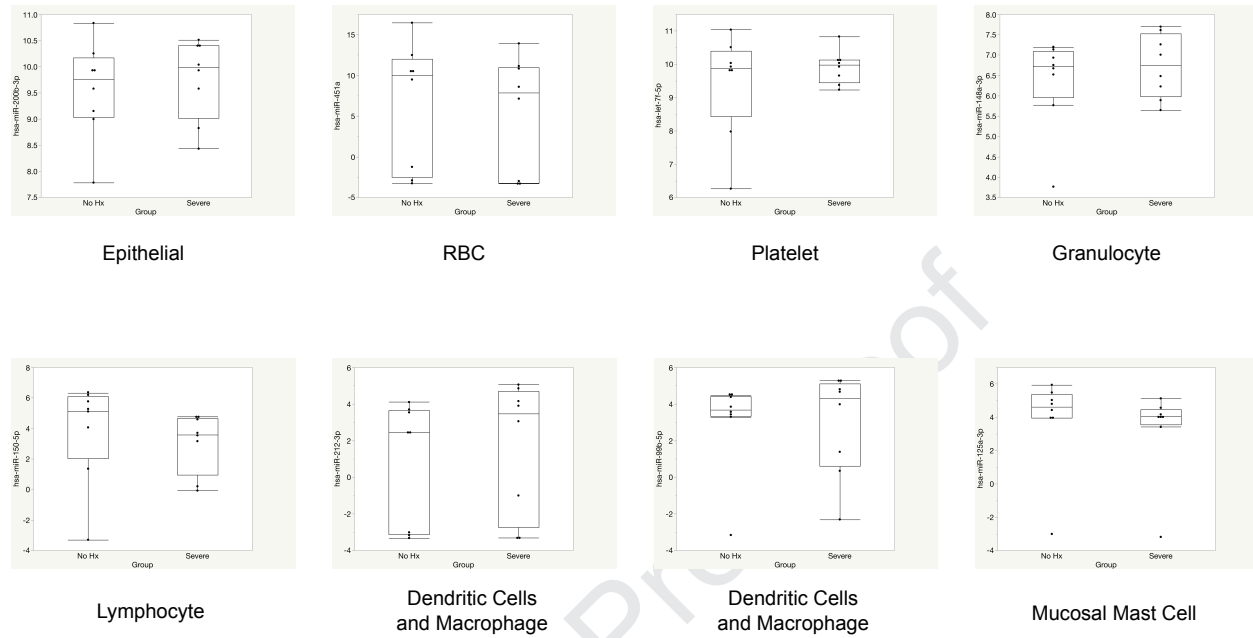
Supplementary Figure E2. Cell types in collected epithelia are consistent in control and severe groups. Expression of microRNAs characteristic for specific cells types was compared between No Hx controls and severe subjects. Significant differences were not observed, suggesting consistency in cell type populations in patient specimens.

Supplementary Figure E1



Supplementary Figure E2

Cell Type Specific microRNAs



Supplementary Methods

Collection and processing of nasal epithelia

Patients were enrolled and samples were collected in the Emergency Department of Children's Hospital of Michigan between June 2012 and June 2013. Nasal epithelia were collected using the Rhino-Pro Curette. Samples were stored at -80°C until they were analyzed for microRNA expression. Total RNA from nasal epithelia was isolated using the "All-in-One Purification Kit" per vendor's protocol (Norgen Biotek Corp). The quantity, purity and quality of the RNA were verified and an RNA integrity number (RIN) was assigned to each specimen. RINs range from 1 to 10, with 10 being the best quality. Samples with a RIN <7.0 ($n=11$) were excluded from subsequent statistical analysis as this reflected RNA degradation.¹

microRNA expression arrays

Expression of microRNAs was measured in each sample using microRNA expression arrays (Agilent G4872A-031181). For each sample, 100ng of total RNA and Agilent microRNA spike-in controls were processed and fluorescently labeled per the vendor's protocol. Hybridization was performed per the vendor protocol and the slides were scanned with an Agilent dual laser scanner. Tiff images were analyzed using Agilent's feature extraction software (11.0.1.1) to obtain fluorescent intensities for each spot on the arrays. Numerous quality control parameters were inspected. The data were imported into GeneSpring (Agilent) for analysis, and probe intensity values were quantile normalized within each microarray.

Statistical analysis of microRNA expression data

Low intensity expression values are the least reliable; therefore we first filtered out microRNA probes in the lower 20 percentile of expression of the overall microarray. This provided 1078 microRNAs and controls (of 1368) for subsequent analysis.

A moderated t-test (GeneSpring) was applied to the normalized expression data for the 1368 microRNAs and used to test for statistical significance between groups. The type I (false positive) error rate was controlled using the Westfall-Young family-wise multiple test correction. A corrected P-value of 0.01 was considered significant. With this stringency, there is only a 1% chance of having one or

more false positives among the microRNAs identified as significant.

Calculation of fold change

Fold-change (FC) is often used in analysis of gene expression data to provide a measure of the change in the expression level of a gene when comparing two conditions. The FC for a given gene is derived from the ratio (R) of the expression level for the gene in the two conditions (A and B), where $R=A/B$. Then $FC = R$ when $R \geq 0$, and $FC = -1/(R)$ when $R < 0$. A log transformation of the expression ratio (e.g. $\log_2 R$) is also a convenient representation since positive values represent an increase in expression and negative values reflect a decrease.²

Cell type analysis

Potential differences in cell type populations were investigated by examining the expression values of microRNAs that are characteristic markers of specific cell types.³⁻⁷ Expression in severe and control groups were compared for the following:

Supplementary References

1. Andreas Schroeder, Odilo Mueller, Susanne Stocker, Ruediger Salowsky, Michael Leiber, Marcus Gassmann, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Molecular Biol. 2006 Jan 31;7(3). PMID: 16448564
2. Yahong Wang, Lawei Yang, Peng Li, Haili Huang, Tie Liu, Huijuan He, et al. Circulating microRNA Signatures Associated with Childhood Asthma. Clin Lab. 2015;61(5-6):467-74. PMID: 26118177
3. Teruel-Montoya R, Kong X, Abraham S, Ma L, Kunapuli SP, Holinstat M, et al. MicroRNA expression differences in human hematopoietic cell lineages enable regulated transgene expression. PLoS One. 2014 Jul 16;9(7):e102259. PMID: 25029370
4. Gilicze AB, Wiener Z, Tóth S, Buzás E, Pállinger É, Falcone FH, et al. Myeloid-derived microRNAs, miR-223,

- miR27a, and miR-652, are dominant players in myeloid regulation. *Biomed Res Int*. 2014;2014:870267. Epub 2014 Aug 11. PMID: 25177699
5. Tserel L, Runnel T, Kisand K, Pihlap M, Bakhoff L, Kolde R, et al. MicroRNA expression profiles of human blood monocyte-derived dendritic cells and macrophages reveal miR-511 as putative positive regulator of Toll-like receptor. *J Biol Chem*. 2011 Jul 29;286(30):26487-95. Epub 2011 Jun 6. PMID: 21646346
 6. Martinez-Anton A, Sokolowska M, Kern S, Davis AS, Alsaaty S, Taubenberger JK, et al. Changes in microRNA and mRNA expression with differentiation of human bronchial epithelial cells. *Am J Respir Cell Mol Biol*. 2013 Sep;49(3):384-95. PMID: 23590309
 7. McCall MN, Kim MS, Adil M, Patil AH, Lu Y, Mitchell CJ, et al. Toward the human cellular microRNAome. *Genome Res*. 2017 Oct;27(10):1769-1781. PMID: 28877962

Supplemental Table 2

microRNA	Cell type
miR-200b-3p	epithelial
let-7f-5p	platelets
miR-451a	red blood cells
miR-148a-3p	granulocytes
miR-150-5p	lymphocytes
miR-212-3p	dendritic and macrophages
miR-99b-5p	dendritic and macrophages
miR-125a-3p	mucosal mast cells

Sample ID	Group	RIN
1011	Severe	9.7
1020	No Hx	9.1
1042	Severe	9.1
1007	No Hx	8.9
1016	Moderate	8.7
1021	Moderate	8.7
1030	No Hx	8.7
1010	Severe	8.7
1001	Hx	8.6
1015	Hx	8.5
1036	Moderate	8.5
1031	Severe	8.5
1002	Severe	8
1027	Hx	7.9
1034	Moderate	7.9
1005	No Hx	7.9
1013	No Hx	7.9
1008	Severe	7.9
1014	Moderate	7.8
1040	No Hx	7.8
1023	Severe	7.8
1004	Hx	7.7
1037	Hx	7.7
1041	Hx	7.7
1006	No Hx	7.7
1022	No Hx	7.7
1048	Severe	7.7
1017	Moderate	7
1018	Severe	6.9
1026	Hx	6.8
1003	No Hx	6.8
1044	Hx	6.7
1043	Hx	6.5
1033	Moderate	6.3
1012	Moderate	6.2
1029	Moderate	6.2
1039	No Hx	6.2
1038	Hx	5.9
1045	Moderate	5.9