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Running title: Salivary protein biomarkers for asthma

A shotgun proteomic approach reveals novel potential salivary protein biomarkers for asthma

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ABSTRACT

Objective: The aim of this study was to determine if there is an association between the salivary protein profile and disease control in asthma.

Methods: Thirty asthmatic patients (17 adults and 13 children) participated in this study. Saliva samples were collected from healthy subjects, controlled and uncontrolled asthmatics. Individual samples from each group were combined to form a pooled sample, from which proteomic analysis was performed using gel-based quantitative proteomics.

Results: Fourteen out of thirty asthmatics were classified to be controlled asthma. Most of asthmatics received inhaled corticosteroids as the controller medications. SDS-PAGE showed predominant bands at high molecular weight in asthmatic saliva compared to that of the controls. Shotgun proteomic analyses indicated that 193 salivary proteins were expressed in both controlled and uncontrolled asthmatics. They were predicted to associate with proteins involved in pathogenesis of asthma including IL-5, IL-6, MCP-1, VEGF, and periostin and asthma medicines (Cromolyn, Nedocromil, and Theophylline). Nucleoside diphosphate kinase (NME1-NME2) only expressed in controlled asthmatics whereas polycystic kidney and hepatic disease 1 (PKHD1)/fibrocystin, zinc finger protein 263 (ZNF263), uncharacterized LOC101060047 (ENSG00000268865), desmoglein 2 (DSG2) and S100 calcium binding protein A2 (S100A2) were only found in uncontrolled asthma. Therefore, the six proteins were associated with disease control in children and adults with asthma.

Conclusion: Our findings suggest that NME1-NME2, PKHD1, ZNF 263, uncharacterized LOC101060047, DSG 2 and S100 A2 in saliva are associated with disease control in asthma.

KEYWORDS: asthma; saliva; shot gun proteomics; protein biomarkers; STITCH

Introduction

Asthma is a common chronic respiratory disease which affects the lives of millions of people worldwide [1]. Tissue-specific diagnostic methods including bronchoalveolar lavage (BAL), sputum, nasal lavage fluid (NFL), bronchoscopy, or bronchial biopsy, are used to measure airway inflammation and remodeling, and provide reliable and detailed clinical information of asthmatic patients. However, the invasiveness of these diagnostic procedures limits the use of these methods for daily clinical routine in most asthmatics [2]. Human saliva is an attractive body fluid for disease diagnosis and prognosis because saliva testing is simple, safe, low-cost and noninvasive [3].

Increased levels of Interleukin 5 (IL-5), IL-6, monocyte chemoattractant protein-1 (MCP-1) or C-C motif chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF) and periostin were found in serum and saliva of asthmatic patients [4-7] which indicates that the proteins are involved in the pathogenesis of asthma. Hence, our study aims to compare salivary protein profile in asthmatic patients at different levels of asthma control and treatment using a proteomic approach.

Materials and Methods

Participant demographics and clinical characteristics

The protocol for this study was approved by Ethical committee of Thammasat University (MTU-EC-PE-6-034/60) and informed consent was obtained. For our proteomic study, a sample size calculation was based on a total biological variation of 40%, a power of 80%, an alpha equal to 0.05 and cut-off of at least 2-fold changes. The minimal subject number of each group was 11 [8]. Thirty asthmatic patients (17 adults and 13 children) were enrolled from the asthma clinic, Thammasat University hospital. Inclusion criteria: children and adults diagnosed with asthma for at least 6 months were included in this study. All patients underwent a detailed history, physical examination, allergy testing and assessment of asthma control. Skin prick testing with 6 aeroallergens including house dust mite, cockroach, grass, cat, dog and mold (ALK-Abello', Horsholm, Denmark) was performed. Demographic data, co-morbidities, treatment medications and level of asthma control according to Global Initiative of Asthma guideline (GINA) were assessed [9]. Fifteen healthy volunteers not taking any medications were recruited from school children and check-up clinic. All subjects were non-smokers, and those suffering from other chronic diseases were excluded.

Saliva collection

Unstimulated whole saliva samples were collected from each subject after a 30 min period of food or liquid abstinence. The subjects were asked to drool out saliva into a 50 ml polystyrene tube (Falcon conical centrifuge tube, Corning, Tewksbury, MA) and kept on ice within 3 min. The saliva samples were centrifuged at 1000 ×g for 20 min at 4°C to remove food remnants, insoluble materials and cell debris. The resulting supernatant was divided into aliquots and stored at -80°C until analysis.

Summary of proteomic workflow

An overview of the workflow is presented in Figure 1. Proteomic analyses were carried out by following the procedures described previously [10]. To diminish the influences of between-individual variation, the salivary protein samples from healthy individuals, controlled and uncontrolled asthmatic groups were equally pooled respectively. The three pooled samples were analyzed using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE). Protein bands were digested with trypsin and subjected to LC-MS/MS. The resulting spectra were searched for protein identities. DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare, Piscataway, NJ, USA) was used to quantify proteins. The identified and quantified proteins were then analyzed using the Mascot software (Matrix Science, London,

UK). To discover asthma biomarkers, the relationship between protein candidates and asthma pathogenesis related proteins was performed using the Venn, MEV, Panther and STITCH.

One- dimensional gel electrophoresis

Total protein content of the three pooled salivary samples was determined using the method of Lowry *et al* [11] and using bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard. Each sample (50 µg per lane) was migrated on a 12.5% 1D SDS-PAGE as previously described [12]. The gel was silver-stained according to the manufacturer's protocol (Silver-Stained Plus Kit, Bio-Rad, Hercules, CA, USA) and cut into approximately 1mm³ cubes with a clean scalpel for quantitative proteomic analysis.

Analysis of salivary proteins by LC-MS/MS

After SDS-PAGE and cutting of the protein bands, these bands were excised and submitted to an in-gel digestion protocol as previously described [12]. Briefly, gel pieces were dehydrated in 100% acetonitrile, rehydrated in 30 µl of 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate, and incubated for 60 min at room temperature. The DTT solution was removed and the sample alkylated in 30 µl of 100 mM iodoacetamide in 10 mM ammonium bicarbonate in the dark for 60 min at room temperature. The gel pieces were shrunk twice by adding 200 µl of 100% acetonitrile in a shaker at room temperature for 5 min. After the reagent was removed, 20 µl of proteomic grade trypsin solution (Roche Diagnostics, Indianapolis, IN, USA) was added to cover the dry gel pieces and left at room temperature for 20 min. Then 20 µl of 30% acetonitrile was added to cover the gel pieces and kept wet during enzymatic cleavage. Peptides extracted were collected and pooled together in a new test tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

Tryptic peptide samples were injected in triplicate into a HCTUltra LC-MS system (Bruker Daltonics Ltd; Hamburg, Germany) which was coupled with a nanoLC system: UltiMate 3000 LC System (Thermo Fisher Scientific; Madison, WI, USA) as well as an electrospray at the flow rate of 300 nl/min to a nanocolumn (PepSwift monolithic column 100 mm internal diameter 50 µm). A mobile phase of solvent A (0.1% formic acid) and solvent B (80% acetonitrile and 0.1% formic acid) were used to elute peptides using a linear gradient of 10-70% of solvent B at 0-13 min (the time-point of retention) followed by 90% B at 13-15 min to remove all peptides in the column. The final elution of 10% B at 15-20 min was carried out to remove any remaining salt. Peptide fragment mass spectra were acquired in data-dependent AutoMS mode with a scan range of 300–1500 *m/z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50–3000 *m/z*. [12, 13].

Protein quantitation and identification

For the quantitation of proteins, DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) was used. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignments, and quantitation based on peptide-ion signal intensity in MS mode. The analyzed MS/MS data from DeCyderMS was submitted to database search using the Mascot software (Matrix Science, London, UK). The data was searched against the NCBI database for protein identification. Identified proteins had at least two peptides with an individual mascot score corresponding to $P < 0.05$ and $P < 0.1$, respectively [12]. Gene ontology annotations including biological process, cellular component and molecular function were performed using Panther database [14]. Protein-chemical interactions were analyzed according to STITCH 5 [15].

Statistical analysis

Salivary protein profiles were analyzed using an Analysis of Variance (ANOVA) to determine the difference(s) among three groups at a significance level of 0.05. A minimum of a 2-fold change and false discovery rate (FDR) < 0.05 were considered statistically significant in proteomic analysis.

Results

Demographic and clinical data of the asthmatic patients and controls

The age of asthmatics ranged between 4-63 years and majority of patients were female (60%). Twenty two out of thirty asthmatic patients were positive to allergy skin testing, most commonly being house dust mite. We found that 46.67% of patients were assessed to be controlled asthma and most of the patients received controller medications. The demographic and clinical data of the asthmatic patients and controls are shown in Table1.

Proteomic analyses

SDS-PAGE shows predominant bands at high molecular weight in asthmatic saliva compared to that of the controls (Figure 2). A total of 1,691 differentially expressed proteins in saliva samples across the three groups were identified. Mostly they were involved in cellular process, biological regulation and metabolic process with percentages of 23.3%, 15.5-16.5% and 15.7-16.1%, respectively. However, there was no statistically significant difference among the three groups in terms of biological functions ($P > 0.05$) (Figure 3A). For gene ontology analysis, a minimum of a 2-fold change and FDR < 0.05 in group comparison were allowed for the first screening of human protein lists. The Venn diagram of these proteins indicated that one protein was found in controlled asthma while 193 proteins were presented in both controlled and uncontrolled asthma. Five proteins were exclusively detected in uncontrolled asthma (Figure 3B). Table 2 shows the protein lists that were found only in controlled asthma, uncontrolled asthma as well as in both groups.

To predict the potential functional interactions among 193 proteins detected in both controlled and uncontrolled asthmatics, the network based on data from the STITCH 5 online database was analyzed. Only 137 proteins out of 193 proteins matched with proteins in database. Furthermore, the network has been expanded by an additional 10 most related proteins based on high-throughput text-mining [16]. Figure 4 shows a network consisting of 154 nodes and 201 interaction edges which were predicted to associate with proteins involved in pathogenesis of asthma including IL-5, IL-6, MCP-1/CCL2, VEGF, and periostin; and asthma medicines (Cromolyn, Nedocromil, and Theophylline). Statistical enrichment of annotated functions by STITCH 5 showed that vascular endothelial growth factor receptor signaling pathway, positive regulation of endothelial cell proliferation, cellular response to organic substance, TOR signaling and enzyme linked receptor protein signaling pathway are predicted to be involved in pathogenesis of asthma (FDR = 0.00144 - 0.05).

We found one protein i.e. nucleoside diphosphate kinase (NME1-NME2) only expressed in controlled asthma and it showed the association with the proteins and drugs involved in asthma (Figure 5). Five salivary proteins including polycystic kidney and hepatic disease 1 (PKHD1), zinc finger protein 263 (ZNF263), uncharacterized LOC101060047 (ENSG00000268865), desmoglein 2 (DSG2) and S100 calcium binding protein A2 (S100A2) were only expressed in uncontrolled asthmatics however they were not apparently involved in any established interaction with proteins and medicines involved in pathogenesis of asthma (Figure 6). Therefore, our findings suggest that the five proteins are the novel proteins involving in uncontrolled asthma.

Discussion

In our study, a comparative proteomic analysis was performed between healthy and asthmatic groups to investigate alterations in salivary proteins in asthma.

We identified 1,492 proteins in the controls; significantly more than a previous report [17]. The higher number of salivary protein identification in the healthy subjects of our study is probably due to the differences in sampling methods, grouping methods, saliva-based proteomic approaches and the subjects included in studies. Based on gene ontology analysis, most of the proteins that were identified as related to biological functions were involved in cellular process, biological regulation and metabolic process. However the distribution of salivary proteins according to functional characteristic was in line with a previous study [10].

STITCH 5.0 was used to identify binding partners for salivary inflammatory mediators (IL-5, IL-6, MCP-1/CCL2, VEGF, and periostin) in asthma and to generate a protein interaction network in this study. Our data indicated that salivary nucleoside diphosphate kinase, fibrocystin, zinc finger protein 263, uncharacterized LOC101060047 (ENSG00000268865), desmoglein 2 and S100A2 were associated with asthma. To the best of our knowledge, it is the first evidence that the six salivary proteins involve in disease control in asthma [18-20].

Nucleoside diphosphate kinases are multifunctional proteins encoded by the *nme* (non-metastatic cells) genes, also called NM23 [21]. Preliminary data suggests that it co-localized with the cystic fibrosis protein (CFTP) and plays a role in ATP production in airway epithelia [22]. Simulation with ATP led to production of mucin (MUC5AC) in human airway epithelial cell lines [23].

Concerning fibrocystin, one study showed that it may participate in regulating mucocilliary sensing and transport within pulmonary airways [24]. In addition, chronic lung disease was observed in 11% of surviving autosomal recessive polycystic kidney disease (ARPKD) patients, suggesting a direct role of fibrocystin in normal lung epithelial cell functions [25]. Desmoglein-2 is a secreted intercellular desmosome junction protein that functions in cellular adhesion. This protein may be vital to maintaining the homeostatic barrier function of airway epithelia [26]. It is suggested that the alteration of this protein may involve in asthma, where the barrier function of epithelium is intrinsically disrupted [27].

S100A2 is a member of a family of the S100/calgranulin Ca^{++} -binding proteins implicated in a number of inflammatory diseases [28]. Little is known about the role of S100A2 in human asthmatics compared to S100A8/A9 [29, 30] and S100A12 [31, 32]. *S100A2* gene was less upregulated in bronchial epithelial cells of asthmatic patients than normal subjects in response to ozone exposure and virus infection [33]. Little is known about the function of ZNF263, except that it may have a repressive effect on gene transcription and often binds intragenic regions [34]. ZNF263 is involved in the development of the many complex diseases associated with stress and aging [35].

It is worth noting that there are several limitations to our study. First, the pediatric and adult studies were not compared with regards to salivary protein profiles. While this limited our ability to compare salivary protein profiles between adult and pediatric patients, there were meaningful clinical parameter differences in marker profiling between controlled and uncontrolled asthmatics. Although there was a difference in phenotypes and endotypes between childhood and adult asthma [36, 37], the expression of asthma associated proteins namely IL-5, IL-6, MCP-1, VEGF and periostin used in our shotgun proteomic approach was enhanced in both types of asthmatic patients [4-7, 38]. Secondly, while sample sizes in our study seem to be small, we found a significant difference with $\text{FDR} < 0.05$ in certain protein expression between groups. For proteomic analysis, we used the cut-off of ≥ 2 fold-change which was classified as a large effect size. Therefore small sample sizes were required to detect significant differences [39]. In addition, it has been suggested that the rate of false positive is not dependent on sample size [40, 41]. Thus, the sample size in our study was sufficient to provide significance at the 0.05 level with 80% power. Nevertheless, this sample size was more or

less the same as the related previous studies [10, 42]. Thirdly, while our results permit drawing conclusions regarding asthmatic disease control based on salivary profiling, no significant difference was found in the protein profiles between healthy subjects and asthmatic patients. Therefore, our findings do not permit to identify potential biomarkers for asthma.

Taken together, our observations support previous studies that patient-based saliva proteomics is a promising approach to the discovery of biomarkers for asthma detection. Although our study bears some limitation as mentioned above but our results obtained by shotgun proteomics suggest that the six salivary proteins as such are associated with disease control in children and adults with asthma. However, protein quantification as additional validation of these biomarkers will need to be carried out in the saliva of asthmatics.

Conclusion/key findings

Our proteomic analysis suggests that nucleoside diphosphate kinase, PKHD1, zinc finger protein 263, uncharacterized LOC101060047 (ENSG00000268865), desmoglein 2 and S100A2 in saliva are related to disease control in children and adults with asthma.

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Disclosure statement

The authors have no financial, consulting or personal relationship(s) disclosures to report.

References

1. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, Williams H, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC phases one and three repeat multicountry cross-sectional surveys. *Lancet* 2006;368: 733-743.
2. Vijverberg SJ, Hilvering B, Raaijmakers JA, Lammers JW, Maitland-van der Zee AH, Koenderman L. Clinical utility of asthma biomarkers: From bench to bedside. *Biologics* 2013;7: 199-210.
3. Hu S, Loo JA, Wong DT. Human salivary proteomic analysis and disease biomarker discovery. *Expert Rev Proteomics* 2007;4: 531-538.

4. Little FF, Delgado DM, Wexler PJ, Oppenheim FG, Mitchell P, Feldman JA, Walt DR, et al. Salivary inflammatory mediator profiling and correlation to clinical disease markers in asthma. *PLoS One* 2014;9: e84449.
5. Hachim MY, Elemam NM, Ramakrishnan RK, Hachim IY, Salameh L, Mahboub B, Al Heialy S, et al. Confounding patient factors affecting the proper interpretation of the periostin level as a biomarker in asthma development. *J Asthma Allergy* 2020;13: 23-37.
6. Liang Z, Liu L, Zhao H, Xia Y, Zhang W, Ye Y, Jiang M, et al. A systemic inflammatory endotype of asthma with more severe disease identified by unbiased clustering of the serum cytokine profile. *Medicine (Baltimore)* 2016;95: e3774.
7. Matsumoto H. Serum periostin: A novel biomarker for asthma management. *Allergol Int* 2014;63: 153-160.
8. Levin Y. The role of statistical power analysis in quantitative proteomics. *Proteomics* 2011;11: 2565-2567.
9. GINA. Global strategy for asthma. Global strategy for asthma management and prevention. Global initiative for asthma. 2020.
10. Sanguansermsri P, Jenkinson HF, Thanasak J, Chairatvit K, Roytrakul S, Kittisenachai S, Puengsurin D, et al. Comparative proteomic study of dog and human saliva. *PLoS One* 2018;13: e0208317.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193: 265-275.
12. Laothumthut T, Jantararat J, Paemanee A, Roytrakul S, Chunhabundit P. Shotgun proteomics analysis of proliferating STRO-1-positive human dental pulp cell after exposure to nacreous water-soluble matrix. *Clin Oral Investig* 2015;19: 261-270.
13. Janan M, Proungvitaya S, Limpaboon T, Proungvitaya T, Roytrakul S, Wongkham C, Jearanaikoon P, et al. Serum adhesion molecule-1 (ICAM-1) as a potential prognostic marker for cholangiocarcinoma patients. *Asian Pac J Cancer Prev* 2012;13 Suppl: 107-114.

14. Mi H, Muruganujan A, Huang X, Ebert D, Mills C, Guo X, Thomas PD. Protocol update for large-scale genome and gene function analysis with the PANTHER classification system. *Nat Protoc* 2019;14: 703-721.
15. Szklarczyk D, Santos A, von Mering C, Jensen LJ, Bork P, Kuhn M. STITCH 5: Augmenting protein-chemical interaction networks with tissue and affinity data. *Nucleic Acids Res* 2016;44: D380-384.
16. Kuhn M, von Mering C, Campillos M, Jensen LJ, Bork P. STITCH: Interaction networks of chemicals and proteins. *Nucleic Acids Res* 2008;36: D684-688.
17. Ventura T, Ribeiro NR, Dionizio AS, Sabino IT, Buzalaf MAR. Standardization of a protocol for shotgun proteomic analysis of saliva. *J Appl Oral Sci* 2018;26: e20170561.
18. Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S. Defining salivary biomarkers using mass spectrometry-based proteomics: A systematic review. *OMICS* 2011;15: 353-361.
19. Al Kawas S, Rahim ZH, Ferguson DB. Potential uses of human salivary protein and peptide analysis in the diagnosis of disease. *Arch Oral Biol* 2012;57: 1-9.
20. Katsani KR, Sakellari D. Saliva proteomics updates in biomedicine. *J Biol Res (Thessalon)* 2019;26: 17.
21. Attwood PV, Muimo R. The actions of NME1/NDPK-A and NME2/NDPK-B as protein kinases. *Lab Invest* 2018;98: 283-290.
22. Muimo R, Crawford RM, Mehta A. Nucleoside diphosphate kinase a as a controller of AMP-kinase in airway epithelia. *J Bioenerg Biomembr* 2006;38: 181-187.
23. Kim K, Kim HJ, Binas B, Kang JH, Chung IY. Inflammatory mediators ATP and S100A12 activate the nlrp3 inflammasome to induce MUC5AC production in airway epithelial cells. *Biochem Biophys Res Commun* 2018;503: 657-664.
24. Hu Q, Wu Y, Tang J, Zheng W, Wang Q, Nahirney D, Duszyk M, et al. Expression of polycystins and fibrocystin on primary cilia of lung cells. *Biochem Cell Biol* 2014;92: 547-554.
25. Guay-Woodford LM. Murine model of polycystic kidney disease: Molecular and therapeutic insights. *Am J Physiol Renal Physiol* 2003;285: F1034-F1049.

26. Pillai DK, Sankoorikal BJ, Johnson E, Seneviratne AN, Zurko J, Brown KJ, Hathout Y, et al. Directional secretomes reflect polarity-specific functions in an in vitro model of human bronchial epithelium. *Am J Respir Cell Mol Biol* 2014;50: 292-300.
27. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, Haitchi HM, et al. Defective epithelial barrier function in asthma. *J Allergy Clin Immunol* 2011;128: 549-556 e541-512.
28. Sedaghat F, Notopoulos A. S100 protein family and its application in clinical practice. *Hippokratia* 2008;12: 198-204.
29. Halayko AJ, Ghavami S. S100A8/A9: A mediator of severe asthma pathogenesis and morbidity? *Can J Physiol Pharmacol* 2009;87: 743-755.
30. Lee TH, Jang AS, Park JS, Kim TH, Choi YS, Shin HR, Park SW, et al. Elevation of S100 calcium binding protein A9 in sputum of neutrophilic inflammation in severe uncontrolled asthma. *Ann Allergy Asthma Immunol* 2013;111: 268-275 e261.
31. Yang Z, Yan WX, Cai H, Tedla N, Armishaw C, Di Girolamo N, Wang HW, et al. S100A12 provokes mast cell activation: A potential amplification pathway in asthma and innate immunity. *J Allergy Clin Immunol* 2007;119: 106-114.
32. Camoretti-Mercado B, Karrar E, Nunez L, Bowman MA. S100A12 and the airway smooth muscle: Beyond inflammation and constriction. *J Allergy Ther* 2012;3: S1-007.
33. Agrawal A, Sinha A, Ahmad T, Aich J, Singh P, Sharma A, Ghosh B. Maladaptation of critical cellular functions in asthma. *Bioinform anal Physiol Genomics* 2009;40: 1-7.
34. Natt D, Johansson I, Faresjo T, Ludvigsson J, Thorsell A. High cortisol in 5-year-old children causes loss of DNA methylation in sine retrotransposons: A possible role for ZNF263 in stress-related diseases. *Clin Epigenetics* 2015;7: 91.
35. Januar V, Saffery R, Ryan J. Epigenetics and depressive disorders: A review of current progress and future directions. *Int J Epidemiol* 2015;44: 1364-1387.
36. Eze J, Uwaezuoke S. Childhood and adult asthma: Phenotype- and endotype-based biomarker. In: Pereira C, editor: *Asthma: Biological Evidence*. Rijeka, Croatia: InTechOpen 2019.

- 37.Tabatabaian F. Asthma phenotypes and biomarkers. In: Mahmoudi M, editor: Allergy and Asthma. Switzerland: Springer Nature; 2019: 275-288.
- 38.Habernau Mena A, Del Pozo Abejon V, Rodriguez Vidigal FF, Bobadilla Gonzalez P. Role of periostin in uncontrolled asthma in children (DADO study). J Investig Allergol Clin Immunol 2017;27: 291-298.
- 39.Dalman M, Deeter A, Nimishakavi G, Duan Z-H. Fold change and p-value cutoffs significantly alter microarray interpretations. BMC Bioinform 2012;13: S11.
- 40.Curran-Everett D. Corp: Minimizing the chances of false positives and false negatives. J Appl Physiol 2017;122: 91-95.
- 41.de Winter J. Using the students t-test with extremely small sample sizes. Pract Assess Res Eval 2013;18: Article 10.
- 42.Talungchit S, Buajeeb W, Lerdtripop C, Surarit R, Chairatvit K, Roytrakul S, Kobayashi H, et al. Putative salivary protein biomarkers for the diagnosis of oral lichen planus: A case-control study. BMC Oral Health 2018;18: 42.

Table 1 Demographic and clinical data of the asthmatic patients and controls

Variable Asthmatic patients Controls (N=11)			
	Children (N=13) Adult (N=17)		
<i>Age (N, column %)</i>	<5y (3, 23.08%)	16 <30y (4, 23.53%)	<15y (7, 63.64%)
	5 < 10y (7, 53.85%)	30 < 50y (6, 35.29%)	> 30-50y (3, 27.27%)
	10-15y (3, 23.08%)	50-70 y (7, 41.18%)	>50y (1, 9.09%)
<i>Gender</i>			
<i>Male [N (column %)]</i>	9 (69.23%)	3 (17.65%)	7(63.64%)
<i>Female [N (column %)]</i>	4 (30.77%)	14 (82.35%)	4 (36.36%)
<i>Positive skin prick test [N (%)]</i>	11 (84.62%)	11 (64.70%)	N/A
<i>Asthma Controlled [N (%)]</i>	6 (46.15%)	8 (47.06%)	N/A
<i>Step of asthma treatment*</i>			
<i>[N (column %)]</i>			
-Step 1	3 (23.08%)	0 (0%)	N/A
-Step 2	3 (23.08%)	3 (17.65%)	N/A
-Step 3	5 (38.46%)	7 (41.18%)	N/A
-Step 4	2 (15.38%)	6 (35.29%)	N/A
-Step 5	0 (0%)	1 (5.88%)	N/A

*Step of asthma treatment: Step 1: as needed rapid acting beta2-agonist (RABA), Step 2: low dose inhale corticosteroids (ICS), Step 3: low dose ICS+long acting beta2-agonist (LABA), Step 4: medium dose ICS+LABA and add on medications (antileukotrienes or theophylline) and Step 5: medium dose ICS+LABA, add on medications and Anti-IgE. N/A= information not available

Table 2 List of salivary proteins detected only in controlled asthma, uncontrolled asthma as well as in both groups

Accession number	Protein name	Accession number	Protein name
<i>Proteins detected only in controlled asthma (1)</i>			
O60361	Nucleoside diphosphate kinase		
<i>Proteins detected only in uncontrolled asthma (5)</i>			
P08F94	Fibrocystin	Q8WWR7	Desmoglein 2
B4DI05	Zinc finger protein 263	Q5RHS7	Protein S100-A2
Q6ZV92	uncharacterized protein LOC103263635		
<i>Proteins detected in both controlled and uncontrolled asthma (193)</i>			
Q14257	Reticulocalbin-2	B3KME6	Centrosomal protein 170 kDa
A0A023HHK9	Methylcytosine dioxygenase TET	B3KPL8	Zinc finger protein 559
A0A024QZJ6	Myosin, heavy polypeptide 11, isoform CRA_b	B3KVV1	Thrombomodulin
A0A024R2M8	Xeroderma pigmentosum, complementation group C, isoform CRA_a	B3KXA9	Leucyl-tRNA synthetase
A0A024R394	Cysteine and histidine-rich domain -containing 1, isoform CRA_c	B4DHY3	Breast carcinoma amplified sequence 1
A0A024R5M3	Cortactin, isoform CRA_c	B4DKN4	Caspase-1
A0A024R6S6	Fused toes homolog , isoform CRA_c	B4DKV9	cDNA FLJ60648
A0A024R734	Nuclear factor of activated T-cells 5, tonicity-responsive, isoform CRA_b	B4DLH7	Mannose-P-dolichol utilization defect 1, isoform CRA_c
A0A024R816	Protein tweety homolog	B4DMZ6	Matrix metalloproteinase-23 , mRNA)
A0A024R8D6	Protein kinase C	B4DQ80	Tropomyosin 3, gamma , transcript variant 2, mRNA
A0A075B6Q3	BCL-6 corepressor-like protein 1	B4DR79	Plakophilin-2
A0A075B6Z2	T cell receptor alpha joining 56	B4DRW1	Keratin, type II cytoskeletal 4
A0A087WVZ7	Zinc finger protein 229	B4DSY3	Krueppel-related zinc finger protein 1
A0A087X279	Interferon-induced protein with tetratricopeptide repeats 2	B4DUH8	Carbonic anhydrase
A0A0A0MTB8	WD repeat-containing protein 36	B4DXV1	Elongator complex protein 3 acetyltransferase
A0A0C4DGR5	Zinc finger protein 496	B4DZD2	Coiled-coil domain-containing protein 13
A0A0G2JQ02	Homeobox protein prophet of Pit-1	B4E304	AP-2 complex subunit mu
A0A0U1RQY2	Histone-lysine N-methyltransferase PRDM9	B7Z1S3	Castor homolog 1 zinc finger protein
A0A184AP76	Tumor suppressor RFP2	B7Z829	Endoplasmic reticulum junction formation protein lunapark
A0A1B0GUQ3	Leucine-rich repeat serine/threonine-protein kinase 2	B7Z8H4	Rhomboid 5 homolog 2 , transcript variant 2, mRNA
A0A1B0GVU0	Alpha-aminoacidic semialdehyde dehydrogenase	B7ZMD7	Alpha-amylase
A0A2R8Y534	DnaJ homolog subfamily C member 21	B7ZMF3	MAPK8IP3 protein
A0A2R8YEU8	Kinesin-like protein	B8XCX8	EPC1/ASXL2b fusion protein
A0A2R8YFT0	Oxysterol-binding protein	B8ZZ84	Leucine-rich repeat transmembrane neuronal protein 4
A0A2R8YH14	Amino acid transporter	C9JA91	Transforming acidic coiled-coil-containing protein 3
A0A2U8J906	Ig heavy chain variable region	C9JBL0	Nuclear autoantigen Sp-100
A0A2X0SYS0	ARHGAP5	C9JRR0	Augurin
A0A2Z2CIT2	DNA cytosine-5--methyltransferase 3 beta transcript variant 9	C9JTN9	Collagen alpha-1 chain
A0A384MR25	Epididymis secretory sperm binding protein	C9YSQ4	MHC class I antigen
A0A3B3ISF0	La-related protein 1B	D3DU41	LUC7-like , isoform CRA_a
A0A510GAH8	Nuclear factor kappa-B	D3DU87	CASK interacting protein 1, isoform CRA_a
A0A590UJL7	FERM and PDZ domain-containing protein 4	D3DXI8	ATP receptor
A0A5C2GLZ3	IG c1022 heavy_IGHV1-2_IGHD3-16_IGHJ6	D6RIV3	Zinc finger CCHC domain-containing protein 10
A0A5F9ZH69	SH3 domain-containing protein 21	F1T0J0	Tubby-related protein 1
A2A2M0	Regulation of nuclear pre-mRNA domain-containing protein 1B	F2Z2W7	tRNA -methyltransferase homolog A
A2RRC4	PLA2G4F protein	F4MHG4	Ubiquitously transcribed tetratricopeptide repeat protein Y-linked transcript variant 208
A3R0T7	Liver histone H1e	F5GZ78	Paxillin
A7E234	ZNF644 protein	F5H1K5	DNA repair protein RAD52 homolog
A7LBF9	Sperm associated antigen 17	F5H365	Protein transport protein SEC23
A8K1R1	Receptor expression-enhancing protein	F5H3M3	MANSC domain-containing protein 1
A8K2S4	Non-specific serine/threonine protein kinase	F5H5T6	Uncharacterized protein
A8K6K3	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1, isoform CRA_f	F8VVB8	Limkain-b1
A8K6U2	Stroma HMG-box containing protein	F8W6W8	Regulating synaptic membrane exocytosis protein 2
A8MVJ9	Histone PARYlation factor 1-like	F8WC91	U4/U6.U5 tri-snRNP-associated protein 2
B0IIS4	DNHD1 variant protein	F8WD26	LIM domain only protein 7
H0Y301	Nuclear protein MDM1	H0YBP2	Transcription factor 7
H0Y523	Intersectin-1	H0YE93	Oxysterol-binding protein-related protein 9
H0YFI8	Proline and serine-rich protein 3	H0YLV8	Bloom syndrome protein
H0YQL5	Protein FAM81A	H0YMG0	AN1-type zinc finger protein 6
Accession number	Protein name	Accession number	Protein name
H3BP82	RNA-binding protein with serine-rich domain 1	Q63HQ7	Uncharacterized protein DKFZp686A1736
H3BPF3	E3 ubiquitin-protein ligase ZNF598	Q6GVN4	Hepatocellular carcinoma-associated antigen 59
H3BRF9	Abscission/NoCut checkpoint regulator	Q6LEN0	Prostacyclin synthase
H3BS25	S phase cyclin A-associated protein in the endoplasmic reticulum	Q6P2P2	Protein arginine N-methyltransferase 9
H3BSD9	Unconventional myosin-VIIb	Q6XYC0	LP2209

H3BSZ2	PR domain-containing protein 11	Q71SV8	Crn-related protein kim1
H3BTZ2	Receptor protein serine/threonine kinase	Q7RTV6	Adenylyl cyclase type II
H7BYT7	C-type lectin domain family 1 member B	Q5VVQ1	Translin-associated protein X
H7BZ93	Histone-lysine N-methyltransferase SETD2	Q7Z4F4	MSTP083
H7C0A1	N-glycosylase/DNA lyase	Q7Z4S8	Uncharacterized protein
H7C3J9	GRB2-associated-binding protein 3	Q86UY0	TXNDC5 protein
H7C3Y1	Phospholipase A and acyltransferase 1	Q86W71	GOLGA4 protein
J3JS66	Sodium/potassium-transporting ATPase subunit beta-1-interacting protein 4	Q8IVF2	Protein AHNK2
J3KN19	Coiled-coil domain-containing protein 90B, mitochondrial	Q8IW35	Centrosomal protein of 97 kDa
J3KTK7	Structural maintenance of chromosomes flexible hinge domain-containing protein 1	Q8IXP7	SAMD4B protein
J3QQN7	Lysophospholipase D GDDP1	Q8N2E5	Protein Wnt
J3QQT3	Interleukin-34	Q8N8D7	Sodium/potassium-transporting ATPase subunit beta-1-interacting protein 3
K7ENN9	Polyunsaturated fatty acid lipoxygenase ALOX12	Q8N8P4	cDNA FLJ39068 fis, clone NT2RP7015080
K7EP30	Tubulin polyglutamylase complex subunit 2	Q8N984	cDNA FLJ38235 fis, clone FCBBF2005428
K7EPG1	Activity-dependent neuroprotector homeobox protein 2	Q8NAG4	cDNA FLJ35392 fis, clone SKNSH2000716
K7ERN2	Intercellular adhesion molecule 3	Q8NAV1	Pre-mRNA-splicing factor 38A
L8E7D7	Alternative protein MTAP	Q8NEZ4	Histone-lysine N-methyltransferase 2C
L8EAM5	Alternative protein PKD1L2	Q8TE56	A disintegrin and metalloproteinase with thrombospondin motifs 17
O14610	Guanine nucleotide-binding protein G/G subunit gamma-T2	Q8WVV4	Protein POF1B
O75054	Immunoglobulin superfamily member 3	Q96AX6	DENND1B protein
O75170	Serine/threonine-protein phosphatase 6 regulatory subunit 2	Q96BX8	MOB kinase activator 3A
O75366	Advillin	Q96CH6	MGC20647 protein
O95038	Uncharacterized protein MLL5	Q96DP6	SKIN SECRETORY PROTEIN XP2
P19474	E3 ubiquitin-protein ligase TRIM21	Q96ET8	Golgi apparatus membrane protein TVP23 homolog C
P36888	Receptor-type tyrosine-protein kinase FLT3	Q96GV9	Macrophage immunometabolism regulator
Q12837	POU domain, class 4, transcription factor 2	Q96M42	Putative uncharacterized protein encoded by LINC00479
Q12953	Oxidoreductase	Q96Q27	Ankyrin repeat and SOCS box protein 2
Q13369	RNA binding protein	Q9BRQ8	Ferroptosis suppressor protein 1
Q14644	Ras GTPase-activating protein 3	Q9C0G0	Zinc finger protein 407
Q14C88	AF4/FMR2 family, member 1	Q9H0B8	Cysteine-rich secretory protein LCCL domain-containing 2
Q16513	Serine/threonine-protein kinase N2	Q9H116	GDNF-inducible zinc finger protein 1
Q2M114	INVS protein	Q9H379	Putative uncharacterized protein PRO3102
Q2M5E4	Regulator of G-protein signaling 21	Q9H3A4	PRO2277
Q2NNQ8	FER-1-like protein 4	Q9NYF6	Myotonic dystrophy kinase-related CDC42-binding protein kinase alpha
Q4KKW2	Pancreatic polypeptide receptor 1	Q9NZ20	Group 3 secretory phospholipase A2
Q53QV7	Prolactin releasing hormone	Q9P1K0	PRO1257
Q53SV8	Alpha-1,6-mannosyl-glycoprotein 6-beta-N-acetylglucosaminyltransferase	Q9ULM3	YEATS domain-containing protein 2
Q59F51	F-box protein 10 variant	Q9Y287	Integral membrane protein 2B
Q59G17	N-methyl-D-aspartate receptor subunit 2D variant	Q9Y366	Intraflagellar transport protein 52 homolog
Q5T6Z8	Transmembrane protein 59	U5XN67	SRY-box 9
Q5U000	Cathepsin X	X6R700	Chromatin target of PRMT1 protein
Q5VT61	Tripartite motif-containing 46, isoform CRA_c		

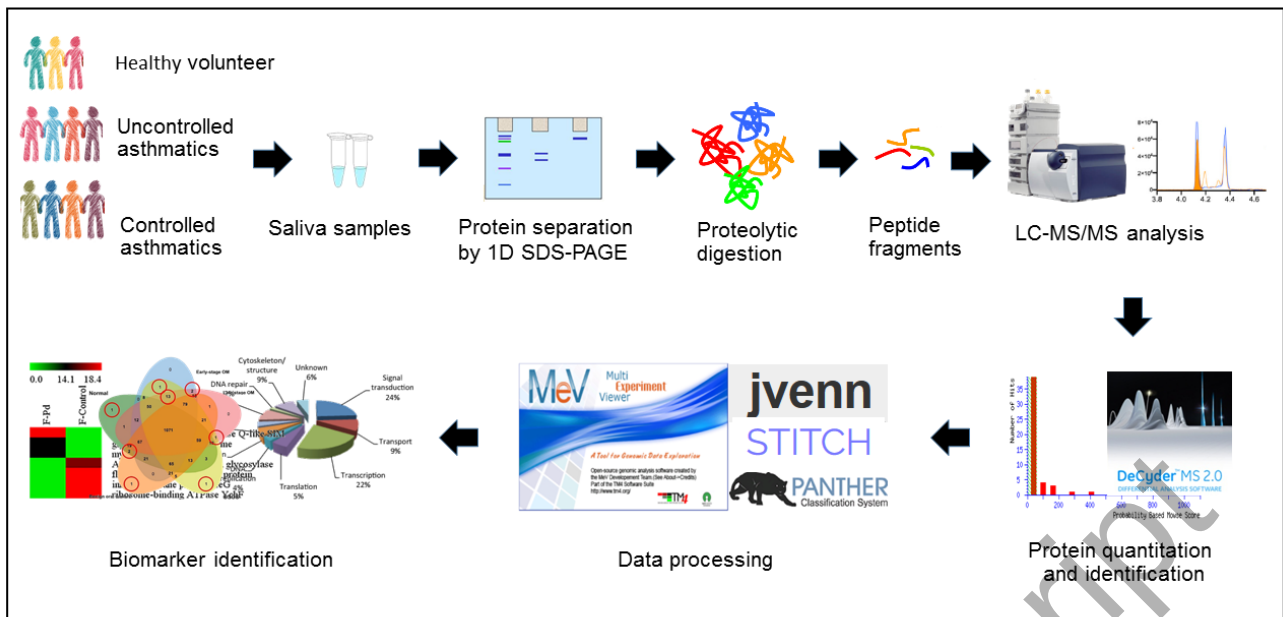


Figure 1. Depicts the workflow for salivary protein biomarker discovery. Saliva samples were collected from participants. Proteins were extracted from the salivary samples, digested into peptides, fragmented by liquid chromatography (LC) and identified by tandem mass spectrometry (MS/MS). Protein quantitation and identification were conducted using DecyderMS and Mascot, respectively. To discover asthma biomarkers, the relationship between protein candidates and asthma pathogenesis related proteins was performed using the Venn, MEV, Panther and STITCH.

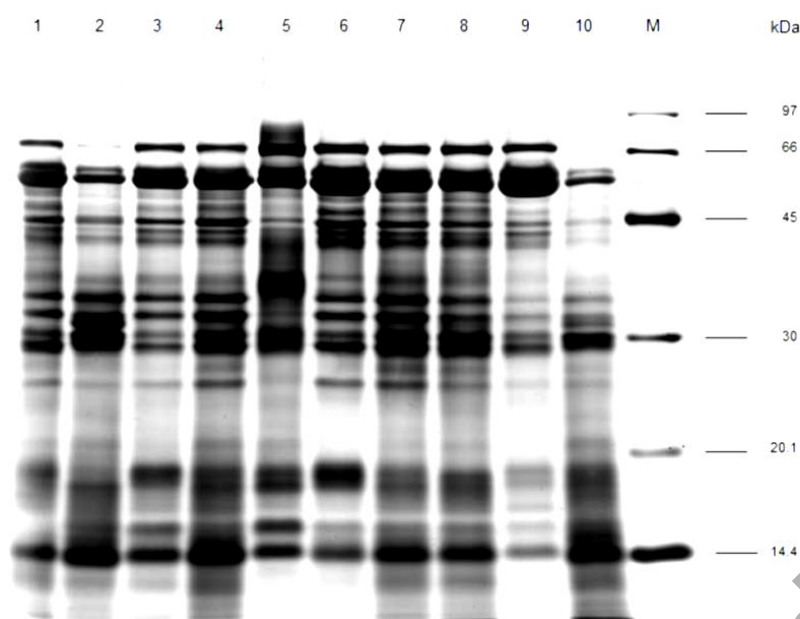


Figure 2 Silver-stained gel of whole saliva samples from asthmatic patients and healthy subjects. Molecular weights of protein marker bands are indicated in kDa on the right of the images. Lane M: LMW-SDS Marker, Lane 1: Saliva from healthy subject, Lane 2-5: Controlled asthma with step of treatment 1-4 (Lane 2 = controlled with step 1, Lane 3= controlled with step 2, Lane 4= controlled with step 3, Lane 5= controlled with step 5) Lane 6-10: Uncontrolled asthma step 1-5.

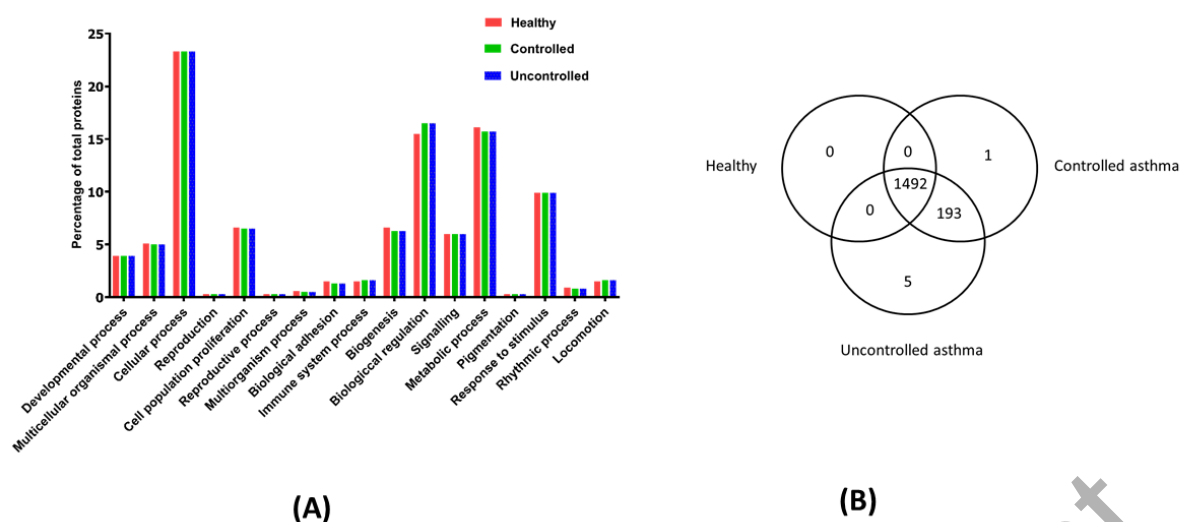


Figure 3 (A) Comparison of functional characteristics of salivary proteins expressed in healthy subjects; control and uncontrolled asthmatics analyzed by Panther. There was no statistically significant difference between the three groups in terms of biological functions ($P>0.05$). **(B)** Intersection of 1,691 differentially expressed proteins in saliva from healthy subject, controlled asthma and uncontrolled asthma by Venn diagram.

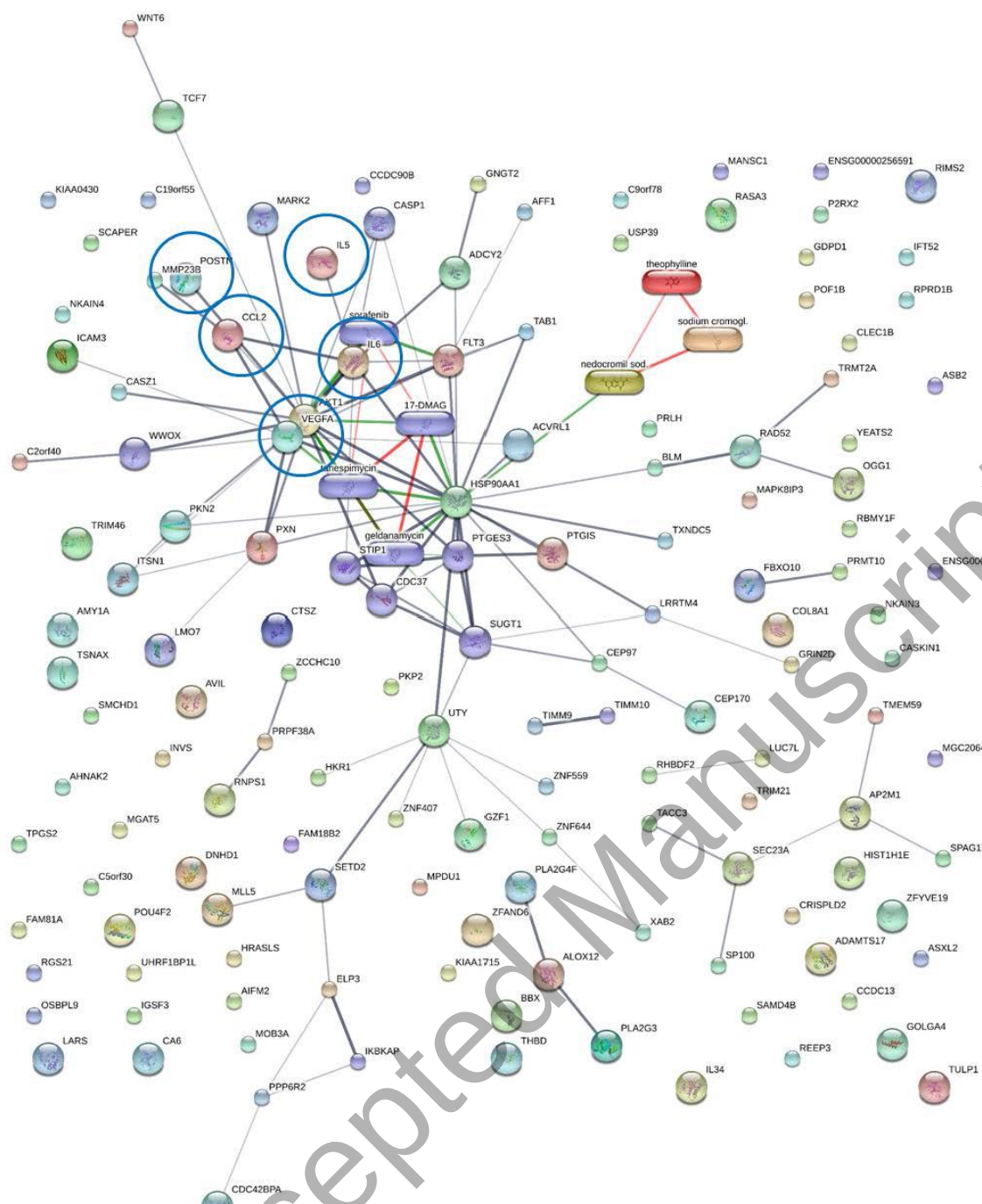


Figure 4 The interaction networks of a protein detected only in asthma patients with proteins involved in pathogenesis of asthma and asthma medicines (Cromolyn, Nedocromil, and Theophylline) by using STITCH version 5. Chemicals are represented as pill-shaped nodes, while proteins are shown as spheres. Blue circle: asthma pathogenesis proteins including IL-5, IL-6, CCL2 (chemokine (C-C motif) ligand 2), VEGFA (vascular endothelial growth factor A) and POSTN (periostin).

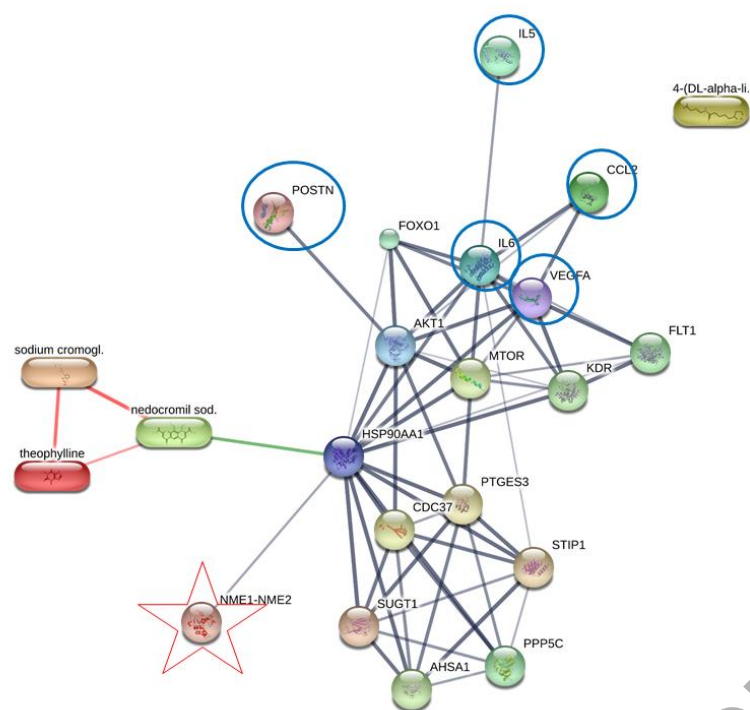


Figure 5 The interaction networks of a protein detected only in controlled asthma with proteins involved in pathogenesis of asthma and asthma medicines (Cromolyn, Nedocromil, and Theophylline) by using STITCH version 5. Chemicals are represented as pill-shaped nodes, while proteins are shown as spheres. Red star: Nucleoside diphosphate kinase (NME1-NME2), blue circle: asthma pathogenesis proteins including IL-5, IL-6, CCL2 (chemokine (C-C motif) ligand 2), VEGFA (vascular endothelial growth factor A) and POSTN (periostin).

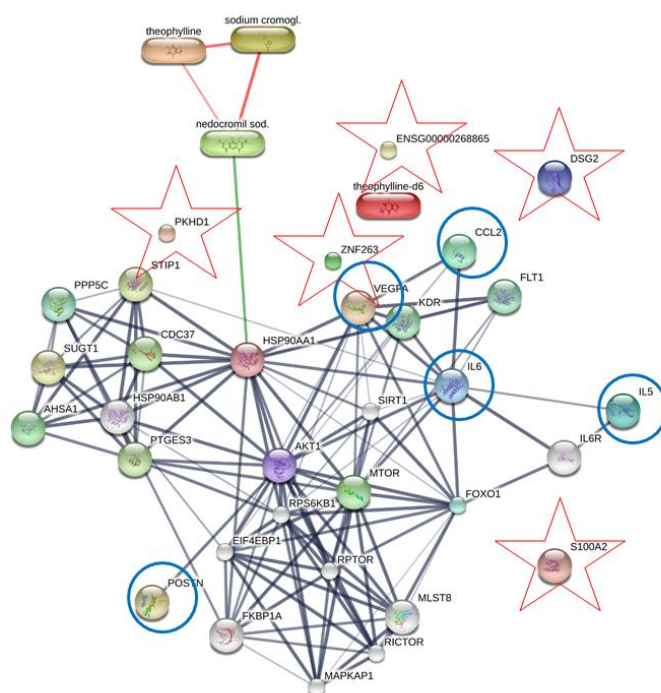


Figure 6 The interaction networks of five proteins detected only in uncontrolled asthma with proteins involved in pathogenesis of asthma and asthma medicines (Cromolyn, Nedocromil, and Theophylline) by using STITCH version 5. Chemicals are represented as pill-shaped nodes, while proteins are shown as spheres. Red star: polycystic kidney and hepatic disease 1 (PKHD1), zinc finger protein 263 (ZNF263), uncharacterized LOC101060047 (ENSG00000268865), desmoglein 2 (DSG2) and S100 calcium binding protein A2 (S100A2), blue circle: asthma pathogenesis proteins including IL-5, IL-6, CCL2 (chemokine (C-C motif) ligand 2), VEGFA (vascular endothelial growth factor A) and POSTN (periostin).