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ORIGINAL RESEARCH

Confounding Patient Factors Affecting the Proper Interpretation of the Periostin Level as a Biomarker in Asthma Development

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Introduction: The proper use of serum periostin (POSTN) as a biomarker for asthma is hindered by inconsistent performance in different clinical settings.
Objective: To explore patient's factors that may affect POSTN expression locally and systematically and its utility as a biomarker for asthma development.
Materials and Methods: Here we used bioinformatics analysis of publicly available transcriptomics data to confirm that *POSTN* is an asthma specific gene involved in core signaling pathways enriched in the bronchial epithelium during asthma. We then explored a large number of datasets to identify possible confounders that may affect the *POSTN* gene expression and consequently, its interpretation as a reliable biomarker for asthma. Plasma and saliva levels of POSTN were determined in locally recruited asthmatic patients (mild, moderate and severe) compared to healthy controls to confirm the bioinformatics findings.
Results: Our bioinformatics results confirmed that *POSTN* was consistently upregulated in the bronchial epithelium in asthma, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF) bronchial epithelium. In asthma, its mRNA expression was affected by gender, sample anatomical site and type, steroid therapy, and smoking. In our cohort, plasma POSTN was upregulated in severe and non-severe asthmatic patients. Saliva POSTN was significantly higher in non-severe asthmatic patients compared to healthy and severe asthmatic patients (specifically those who are not on Xolair (omalizumab)). Patients' BMI, inhaled steroid use and Xolair treatment affected POSTN plasma levels.
Conclusion: Up to our knowledge, this is the first study examining the level of POSTN in the saliva of asthmatic patients. Both plasma and saliva POSTN levels can aid in early diagnosis of asthma. Saliva POSTN level was more sensitive than plasma POSTN in differentiating between severe and non-severe asthmatics. Patients' characteristics like BMI, the use of inhaled steroids, or Xolair treatment should be carefully reviewed before any meaningful interpretation of POSTN level in clinical practice.
Keywords: periostin, biomarkers, transcriptomics

Introduction

Asthma is a heterogeneous disease with diverse etiology resulted from different combinations of environmental, genetic, as well as behavioral risk factors.[1] Asthma heterogeneity is attributable to the various underlying mechanisms, pathways, and mediators,[2] which can be reflected in the ongoing classification of asthma phenotypes.[2] Consequently, there is a continuous effort to refine asthma phenotypes in order to enhance patients' classification and pave the

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way for more precise and personalized therapeutic options.[3] Among many subtypes of asthma, type 2-driven asthma is stratified into "Th2-high" and "Th2-low" asthma.[4] Th2-high asthma has 3 specific gene signatures, namely, periostin (*POSTN*), chloride channel regulator 1 (*CLCA1*), and serpin peptidase inhibitor, clade B, member 2 (*SERPINB2*).[5] Among them, *POSTN* was found to be a more reliable surrogate marker for Th2-high asthma and showed a remarkable performance in predicting the efficacy of anti-IL-13 antibodies (lebrikizumab) for steroid-resistant asthmatic patients.[6] It is widely accepted that under the effect of secreted IL-13 by Th2 immune cells, the human bronchial epithelium will upregulate the expression of *POSTN*.[6] When secreted into the extracellular matrix, POSTN promotes local eosinophil infiltration.[7] This local increase in POSTN level is reflected as an increase in serum POSTN level in adult and children patients with Th2 asthma.[8] The proper use of POSTN as a biomarker is hindered by many limitations as relatively few numbers of proper studies showed conflicting results in different settings.[9] Since POSTN is one of the very few biomarkers available to reflect refractory Th2/eosinophilic inflammation; therefore, understanding the factors that may affect its expression is essential for managing the patients and understanding the disease.[10]

Here we used bioinformatics analysis of publicly available transcriptomics data to confirm that *POSTN* is an asthma specific gene and participate in different pathways in asthmatic patients' bronchial epithelium. Then we explored a large number of datasets to identify possible confounders that may affect *POSTN* gene expression and its use as a potential biomarker. Our approach confirmed that *POSTN* is consistently differentially expressed in a large number of patients across different datasets using bronchial epithelium transcriptomic data. Our analysis showed the effect of gender, sample type, smoking, BMI, and treatment on the expression of *POSTN*. In vivo validation using locally recruited, asthmatic patients confirmed the bioinformatics findings. Saliva POSTN was able to differentiate nonsevere asthmatic from healthy controls. Up to our knowledge, this is the first study examining the level of POSTN in the saliva of asthmatic patients.

Patients, Materials, and Methods

Bioinformatical Approach

Datasets Selection to Identify Common Differentially Expressed Genes (DEG) in Bronchial Epithelium of Asthmatic Patients

Publicly available transcriptomic datasets from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) of asthmatic patients were used for initial analysis. The inclusion criteria for selecting these datasets were as follows: datasets using human samples only, studies that included matching healthy controls, datasets with defined clinical classifications of participants, and datasets with bronchial epithelium gene expression using microarray. In order to have a platform consistency, only datasets generated using Affymetrix Human Genome U133 Plus 2.0 Array were selected. Three datasets fulfilled these criteria: GSE64913, GSE4302, and GSE13396. In total, 190 samples were included in those studies, where 103 asthmatic patients were compared to 71 healthy controls, as shown in Table 1.

Selection of Datasets for Validation of the Generated Common DEG and Identification of Confounding Factors Affecting the Interpretation of Potential Biomarkers

Seven datasets of the same microarray platform were extracted representing different types of samples other than bronchial epithelium such as nasal scraping, sputum, and blood.

Table 1 Details of Datasets Extracted from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) and Used for Initial Identification of DEG Between Asthmatic and Healthy Controls

Geo ID	Title	Total	Asthmatic	Smoker	Healthy
GSE64913	Altered epithelial gene expression in peripheral airways of severe asthma	70	33		37
GSE4302	Genome-Wide Profiling of Airway Epithelial Cells in Asthmatics, Smokers and Healthy Controls	86	42	16	28
GSE13396	Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma	34	28		6
Total	3	190	103	16	71

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Moreover, these datasets included numerous variables such as smoking, steroid inhaler treatment, acute versus convalescent conditions, rhinovirus infection, and exercise-induced bronchospasm, in order to identify the confounding factors that may affect gene expression. Also, we analyzed gene expression that was performed on the different locations of airways epithelium (nasal, central airway and peripheral), as well as gender-specific variations and compared that with other respiratory diseases that share common features with asthma including chronic obstructive pulmonary disease (COPD) and interstitial pulmonary fibrosis (IPF). As shown in Table 2, the total number of explored samples were 615 (263 asthmatics, 36 COPD, 23 IPF, 60 smokers, and 184 healthy controls)

Raw CEL Files Processing, Normalization, and Filtration

In total (n=805) samples were analyzed in two stages, where stage I (n=190) samples were used for identification of common DEG while stage II (n=615) samples were used for the validation. The raw Affymetrix Human Genome U133 Plus 2.0 Array CEL files were extracted, classified into their corresponding clinical group, after which each dataset underwent preprocessing and normalization separately using in house Hamoudi et al[11] R script through R statistical software version 3.0.2. The script used affy, gcRMA, MAS5 and RMA packages for normalization. Hamoudi et al found that the best strategy for generating variant probes that are differentially expressed between the groups was using a combination of gcRMA and MAS5 normalization followed by cross-referencing the probes that passed through non-specific filtering based on the coefficient of variation and absolute value thresholding.[9] After the removal of the non-variant probes, the resultant filtered ones were the only variant probes. Also, the housekeeping probes and those that are not assigned to a gene were excluded. Finally, probes with maximum value were selected among matching probes then collapsed to their corresponding gene using GSEA software (http://software.broadinstitute.org/gsea).

Gene Set Enrichment Analysis (GSEA) to Generate DEG in Each Set

Filtered raw files for each dataset were uploaded to the GSEA software separately, then absolute GSEA (AbsGSEA) was performed to identify the top genes enriched in the asthmatic patients compared to healthy controls in each dataset. Signal to noise metric was used to generate a rank-ordered gene list based on the enrichment score of each gene in the dataset. The enriched genes were filtered based on cutoff score ≥ 0.25 as upregulated genes in asthma, while those ≤ -0.25 as downregulated genes in asthma. For each dataset, the genes that were upregulated or downregulated in asthmatic patients compared to healthy controls were identified and grouped. Subsequently, DEG in each dataset were intersected with the DEG from the other sets, and common genes were identified.

Table 2 Details of Datasets Extracted from Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) and Used for Validation of the Generated Common DEG to Identify Confounding Factors Affecting the Gene Expression

Validation Sets							
Geo ID	**Title**	**Total**	**Asthmatic**	**COPD**	**IPF**	**Smoker**	**Healthy**
GSE41861	Upper airway gene expression is an effective surrogate biomarker for Th2-driven inflammation in the lower airway	138	54				30
GSE41862	Nasal scrape gene expression profiling in asthmatics	116	95				21
GSE16032	Gene expression data from severe asthmatic children: PBMC profiles during acute exacerbation versus convalescence	10	25				
GSE35571	Gene expression data from 131 human subjects in Detroit, Michigan	131	67				64
GSE13785	Novel mediators of eicosanoid and epithelial nitric oxide production in asthma	22	22				
GSE30063	Epithelial Expression of Toll-like Receptor 5 is Modulated in Healthy Smokers and Smokers with Chronic Obstructive Lung Disease	169		36		60	63
GSE21369	Gene expression profiles of interstitial lung disease (ILD) patients	29			23		6
Total	7	615	263	36	23	60	184

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In silico Validation and Identification of Confounding Factors

Raw data of the individual samples' mRNA expression for each of the identified genes were extracted from each dataset in order to confirm the findings of stage I and identify the factors that might affect gene expression level. Next, the resulting short-listed genes were validated using other datasets with samples other than bronchial epithelium, such as nasal epithelium, blood and sputum, and in other respiratory diseases (COPD and IPF).

Enriched Ontology Clustering for the Identified Genes

In order to explore if the identified genes are sharing common pathways, Enriched Ontology Clustering for the identified genes was performed using the Metascape (a web-based tool used for comprehensive gene list annotation and analysis resource) alongside GSEA tool.[12]

Gene-Based Enrichment and Similarity Analysis

In order to perform the correlation of the identified genes as a group to each other, we used eXploring Genomic Relations for enhanced interpretation web tool (http://galahad.well.ox.ac.uk:3040/). This tool can provide enhanced interpretation targeting genomic summary data such as those resulting from genome-wide association studies (GWAS) and expression quantitative trait loci (eQTL) studies via comprehensively utilizing ontology and network information.[13] The tool uses the option "Experimental Factor Ontology (EFO)" that provides a systematic description of many experimental variables available in EBI databases and NHGRI GWAS catalog to show biological ontologies, such as UBERON anatomy, ChEBI chemical compounds, and Cell Ontology.[14] We used Socialiser for Genes Option to assess the degree of relatedness between any two identified genes in the meaning of annotation profiles by ontology terms.

Cell/Tissue-Specific Expression of the Identified Genes

To determine whether the identified genes are tissue-specific to the lung, we uploaded the identified genes to Enrichr open source and freely available online web-based tool (http://amp.pharm.mssm.edu/Enrichr) to generate various types of visualization summaries of collective functions of gene lists.[15] Through Enricher, cell of origin for the given gene list was found by utilizing ARCHS4 resource that makes the majority of published RNA-seq data from humans and mice available at the gene and transcript levels.[16]

Patients Cohort

Subject Characteristics and Study Design

From January 2017 to May 2019, individuals were recruited consecutively from the Asthma Clinic in Rashid Hospital, Pulmonary Medicine Department. 32 asthmatic patients were included, 19 were non-severe asthmatic patients (mild to moderate), and 13 were severe asthmatic subjects (fulfilling the criteria for asthma as per American Thoracic Society). According to GINA and ERS/ATS Task Force on severe asthma, guidelines define asthma severity as "those who require continuous high-dose treatment, as their asthma is refractory or difficult-to-treat plus a second controller (and/or systemic corticosteroids) to prevent it from becoming uncontrolled or which remains uncontrolled despite this therapy".[17] Those patients were compared to 12 non-asthmatic volunteer subjects who had no recent infection of the respiratory tract and no histories of allergy or asthma. Participants completed the Asthma Control Test self-assessment. The Ethics Committee of Dubai Health Authority and the University of Sharjah approved the study with REC (Research Ethics Committee) approval number DSREC-11/2017_04 and each subject gave written informed consent after a thorough explanation by the treating physician and the researchers. This study was conducted in accordance with the Declaration of Helsinki. The demographic characteristics of the asthmatic patients and control subjects are shown in Table 3. Blood and saliva were collected, as previously described.[18]

Plasma Collection Protocol

Peripheral blood was collected in ethylene diamine tetra-acid (EDTA) vacutainer tubes (BD Biosciences, USA). Samples were then centrifuged at 2000 rpm for 15 min; retrieved plasma was stored at −80°C until analysis.

Unstimulated Whole Saliva Collection Protocol

Participants were asked to fast for at least 1 hr and not to brush their teeth or smoke for 30 mins, accompanied by gargling and rinsing of the mouth with water 5 mins before proceeding with saliva collection. 1 mL of unstimulated whole saliva via passive drool was

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Table 3 Characteristics of the Recruited Subjects-Asthmatic Patients and Healthy Controls

	Control	Non-Severe Asthma	Severe Asthma	P-value
Number of subjects	10.00	19.00	13.00	
Age (Years)	37.00±11.73	40.19±15.65	48.42±17.95	NS
Age of Onset (adult to childhood ratio) Childhood Asthma Adult Asthma		 11 5	 5 8	
Female to Male ratio	6:4	13:3	7:6	NS
BMI	24.60±3.19	27.63±6.40	27.03±6.56	NS
ACT Score	–	20.75±4.31	17.55±6.15	NS
Exacerbations/Year	–	1.688±2.575	2.133 ±1.482	NS
History of Atopy Yes No	 0 10	 0 16	 4 9	<0.01
History of Allergic rhinitis Yes No	 1 9	 10 6	 9 4	<0.01
Peak Flow (l/min)	453.80±101.00	299.30±78.20	302.50±139.90	<0.01
FEV1 (% predicted)	–	55.46±42.42	54.06±45.53	NS
FEV1/FVC	–	56.87±40.30	49.13±35.35	NS
Total serum IgE	–	280.30±358.40	1167.00±1428.00	<0.01
Blood Eosinophil (%)	–	4.54±3.24	6.88±9.57	NS
Blood Neutrophil (%)	–	58.29±12.34	60.04±13.86	NS
Blood Basophil (%)	–	0.53±0.36	0.52±0.24	NS
Blood Lymphocytes (%)	–	28.89±11.05	26.67±12.15	NS
Drugs Histoiry during the study				
Oral Steroid Use per day/Week	–	0.6563±1.06	2.083±1.459	0.01
XOLAIR (omalizumab), (n,%)	–	1(5%)	8(66,7%)	<0.01
Montelukast sodium, SINGULAIR, (n,%)	–	10(52.6%)	7(58.3%)	NS
Symbicort (budesonide and formoterol), (n,%)	–	7(36.8%)	6(50%)	NS
Pulmicort (budesonide), (n,%)	–	1(5%)	5(41.6%)	<0.01
Ventolin HFA (albuterol), (n,%)	–	9(52.6%)	3(25%)	NS
Seretide (salmeterol xinafoate/fluticasone propionate), (n,%)	–	7(42.1%)	3(25%)	NS

collected in a pre-prepared 50 mL tube containing 1 mL of RNAlater (Invitrogen). Collected saliva samples were transported on ice and stored at −80°C until analysis. Sample measurements were undertaken within 3 months of storage.

Quantification of POSTN in Plasma and Saliva Using ELISA

POSTN was assessed in the plasma and saliva using Human POSTN/OSF-2 DuoSet ELISA Kit (R&D Systems, USA) according to the manufacturer's instructions. POSTN levels

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were expressed as pg/mL. All assays were performed in duplicates.

Statistical Methods

One-way ANOVA test was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA), to determine whether there are any statistically significant differences between the mean values of the controls and different asthma groups for the gene expression and protein levels. The same software was used to examine the correlations between the different parameters using linear regression. The student's *t*-test was used to look for the difference between two groups under a given experiment or treatment. A p-value <0.05 is taken as statistically significant.

Results

Top Common Genes That are Differentially Expressed in Asthma Compared to Controls

For a better understanding of the genes that are involved in asthma pathogenesis, we initially investigated genes that are differentially expressed in asthmatic patients compared to healthy controls using different datasets. For each dataset, the DEGs were identified and divided into those that are upregulated in asthma (242 genes in GSE4302, 103 genes in GSE64913and 86 genes in GSE13396) and those that are downregulated in asthma compared to healthy (183 genes in GSE4302, 45 genes in GSE64913and 38 genes in GSE13396), as shown in Table 4. A list of all DEGs is provided in Supplementary 1.

Table 4 Total Genes That are Significantly Differentially Expressed Between Healthy and Asthmatic Bronchial Epithelium Categorized into Upregulated and Downregulated Groups

Dataset	Total Genes that are Significantly Differentially Expressed Between Healthy and Asthma	Genes Upregulated in Asthma	Genes Downregulated in Asthma
GSE4302	425	242	183
GSE64913	148	103	45
GSE13396	124	86	38

POSTN and IGF2BP3 are Consistently Enriched in Asthmatic Bronchial Epithelium Samples

DEG of the three datasets were intersected to identify common genes that were differentially expressed between asthma and healthy controls in at least 2 datasets (51 genes) as shown in Figure 1. As illustrated in Figure 1, two genes were found to be enriched in asthmatic bronchial epithelium compared to healthy ones in all of the three training datasets (*POSTN* and *IGF2BP3*), while 49 other genes were differentially expressed in 2 of the three datasets (*KRT6A*, *PHACTR3*, *CXCL14*, *DNAJC12*, *FKBP5*, *SERPINB2*, *CLCA1*, *PRR4*, *PHLDB2*, *TOP2A*, *SRPX2*, *MX2*, *PKP2*, *DQX1*, *GCNT3*, *ATP10B*, *CST1*, *HPGD*, *CD44*, *TMEM106C*, *DPYSL3*, *KIAA1913*, *BIRC5*, *ITPR1*, *PLUNC*, *FLJ21511*, *MUC5B*, *SCNN1G*, *SLAMF7*, *WIF1*, *TACR1*, *HLA-DOA*, *GRP*, *CDH2*, *WNT5A*, *FLJ10781*, *CCNA1*, *IL1RL1*, *SPRR3*, *KRT13*, *DMRT2*, *UPK1B*, *SNORA68*, *IFI44*, *MGC39900*, *MSRB3*, *VGLL3*, *AKAP12*, and *CDC42SE2*).

POSTN Showed a High Degree of Similarity with 14 Out of the 49 Identified DEG

In order to explore the correlation of the identified genes as a group to each other, we used the web tool: eXploring Genomic Relations for enhanced interpretation (http://galahad.well.ox.ac.uk:3020/). Genes with a similarity score of more than 50% were filtered, and the number of related

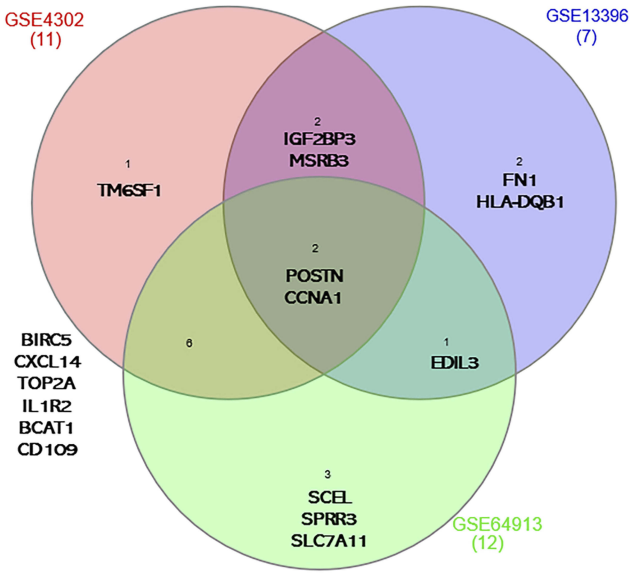


Figure 1 Genes that are differentially expressed in asthma compared to healthy controls in the three datasets GSE64913 (12 genes), GSE4302 (11 genes) and GSE13396 (7 genes). *POSTN* and *IGF2BP3* were consistently differentially expressed among the three transcriptomic datasets.

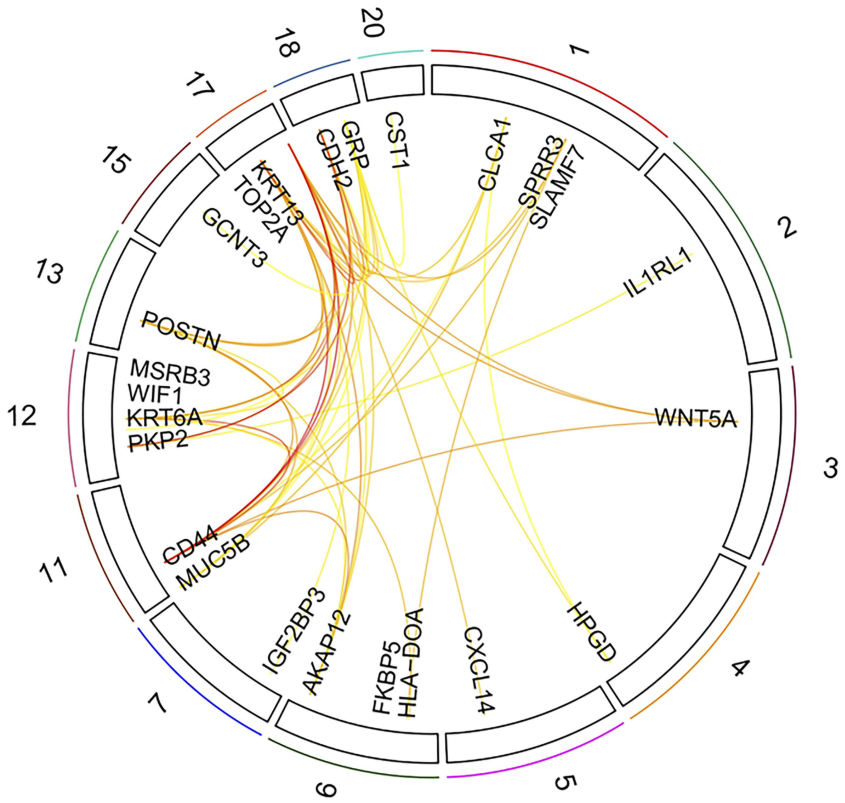
genes in term of annotation were counted for each one of them. *POSTN* exhibited high similarity scores to 14 genes from the identified DEG, and these are *CD44*, *BIRC5*, *MUC5B*, *TOP2A*, *CDH2*, *AKAP12*, *WIF1*, *WNT5A*, *CLCA1*, *GRP*, *HPGD*, *SCNN1G*, *IL1RL1*, and *IGF2BP3*. On the other hand, *IGF2BP3* showed high similarity in the annotation to only 5 genes: TOP2A, CD44, CDH2, AKAP12, and BIRC5, as illustrated in Figure 2. This might indicate that *POSTN* expression can reflect the expression of similar genes that are differentially expressed in asthma and hence can be a candidate biomarker.

The DEG are Enriched in Pathways Related to Response to Wounding, Bacteria, and Regulation of Cytokine Secretion

In order to determine if the identified DEG; common in at least 2 datasets; participate in common pathways, the list was uploaded to metascape (http://metascape.org) to generate a graphical representation of top enriched pathways.[12] As expected, the identified genes were enriched to pathways related to epithelial cells development and physiology (morphogenesis of an epithelial sheet, keratinocyte differentiation, cornification and formation of the cornified envelope) and response to wounding. Interestingly, among the top enriched pathways are immune-related pathways such as response to bacteria, regulation of cytokines and chemokines secretion, and regulation of leukocyte migration, as shown in Figure 3.

The Common DEG Between Asthmatic and Healthy Bronchial Epithelium are Tissue-Specific to Skin, Gut, and Respiratory System

To determine whether the identified genes are lung-specific or are expressed by other tissues, we uploaded the identified genes to the freely available online web-based tool Enrichr open source (http://amp.pharm.mssm.edu/Enrichr) to generate various types of visualization summaries of collective functions of gene lists.[15] Through Enricher, cell of origin for the given gene list was generated by ARCHS4 resource of published RNA-seq data from humans and mice.[16] Our analysis showed that 13–15 of the identified genes are related to lung, skin, and gut. This indicates that tissue sampling other than the respiratory system might reflect



Gene1	Gene2	Similarity
POSTN	CD44	0.806
POSTN	BIRC5	0.798
POSTN	MUC5B	0.777
POSTN	TOP2A	0.776
POSTN	CDH2	0.744
POSTN	AKAP12	0.734
POSTN	WIF1	0.693
POSTN	WNT5A	0.621
POSTN	CLCA1	0.611
POSTN	GRP	0.58
POSTN	HPGD	0.574
POSTN	SCNN1G	0.572
POSTN	IGF2BP3	0.562
POSTN	IL1RL1	0.528
IGF2BP3	TOP2A	0.68
IGF2BP3	CD44	0.647
IGF2BP3	CDH2	0.644
IGF2BP3	AKAP12	0.628
IGF2BP3	BIRC5	0.555

Figure 2 Degree of relatedness of a gene to another gene in the meaning of annotation profiles of the identified genes as a group to each other using eXploring Genomic Relations for enhanced interpretation (http://galahad.well.ox.ac.uk:3024/). *POSTN* showed a high degree of similarity with 14 out of the 49 identified DEG.

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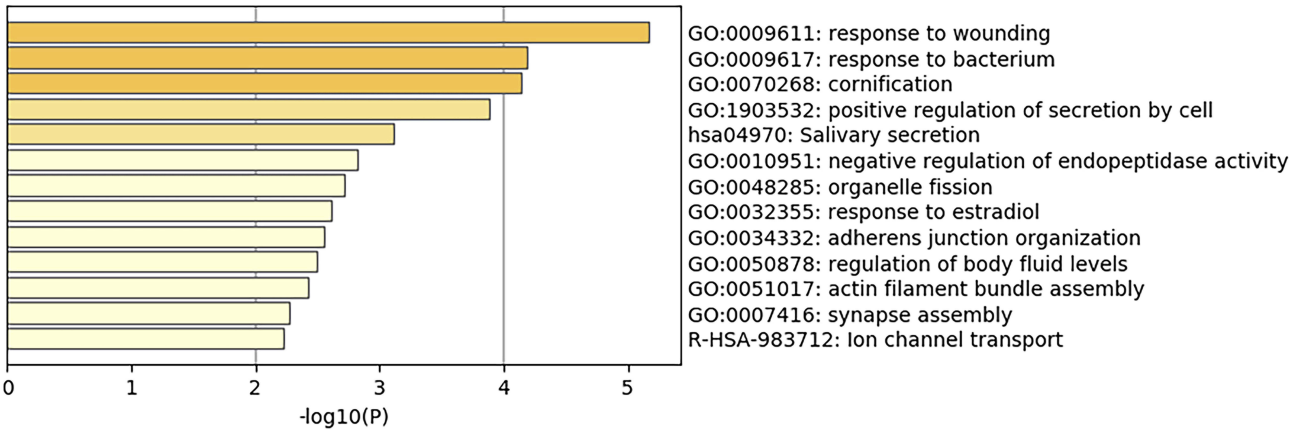

Figure 3 Top pathways shared by the DEGs in at least 2 of the 3 selected datasets. The graph was generated using metascape (http://metascape.org). The DEG are enriched in pathways related to response to wounding, bacteria, and regulation of cytokine secretion.

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expression changes that occur in asthma and can be an alternative for bronchial sampling in the clinical setting.

The DEG are Enriched in Diseases of the Skin, Respiratory and Gut Diseases

In order to determine if the identified DEG common in at least 2 datasets are known markers in any diseases, the gene list was uploaded to eXploring Genomic Relations for enhanced interpretation web tool (http://galahad.well.ox.ac.uk:3040/). Gene-based Enrichment Analysis showed that 10–13 genes in the list are known to be related to phenotypes of intestinal disease, musculoskeletal system disease, connective tissue disease, gastrointestinal system cancer, intestinal cancer, respiratory system disease, large intestine cancer, lower respiratory tract disease, and skin disease. This might indicate that diseases other than the respiratory system might affect gene expression and mimic their potential utilization as useful biomarkers.

Patients Related Factors Like Gender, Smoking or Viral Infection Can Affect the Expression of POSTN

Since *POSTN* was differentially expressed in the 3 datasets of bronchial epithelium between asthmatic patients and healthy controls, we explored its raw mRNA expression in the remaining 7 validation sets along with the three initial datasets. This was performed to identify any confounding variables that may interfere with the proper diagnostic significance of periostin as a biomarker and hence should be taken into consideration in its interpretation.

POSTN Gene mRNA Expression Was Affected by Sample Type, Gender, Use of Flovent Inhaler, Asthma Phenotype, and Its Upregulation Is Not Asthma-Specific

POSTN expression in male's bronchial epithelium was higher compared to females in both central or peripheral airways (Figure 4A). This gender effect on *POSTN* gene expression was only evident in healthy subjects and lost in asthmatic patients (Figure 4B). On the other hand, only females' asthmatic bronchial epithelium expressed higher *POSTN* mRNA in central ($p=0.03$) and peripheral ($p=0.02$) airways than the healthy controls (Figure 4C).

Upper airways nasal epithelium showed higher *POSTN* expression compared to the lower bronchial epithelium ($p<0.01$). Additionally, as shown in Figure 4D, the more peripheral the airways are, the less *POSTN* gene expression ($p<0.01$). Nevertheless, nasal epithelial expression of *POSTN* mRNA was affected by the phenotypes of asthma as it exhibited lower expression in neutrophilic phenotype compared to healthy controls ($p=0.03$) and eosinophilic phenotype ($p=0.02$) as shown in Figure 4E.

Regarding the effect of therapy, it was interesting that fluticasone propionate "Flovent" inhaler significantly downregulated *POSTN* mRNA expression compared to patients at the beginning of the treatment ($p=0.04$) as well as healthy controls ($p<0.01$). As illustrated in Figure 4G, *POSTN* expression was not affected by human rhinovirus infection (HRV) to the bronchial epithelium. Furthermore, *POSTN* was not specific to asthma as it was also upregulated in smokers with COPD ($p=0.04$, Figure 4H) and IPF lung ($p=0.01$, Figure 4I) when compared to matching healthy controls.

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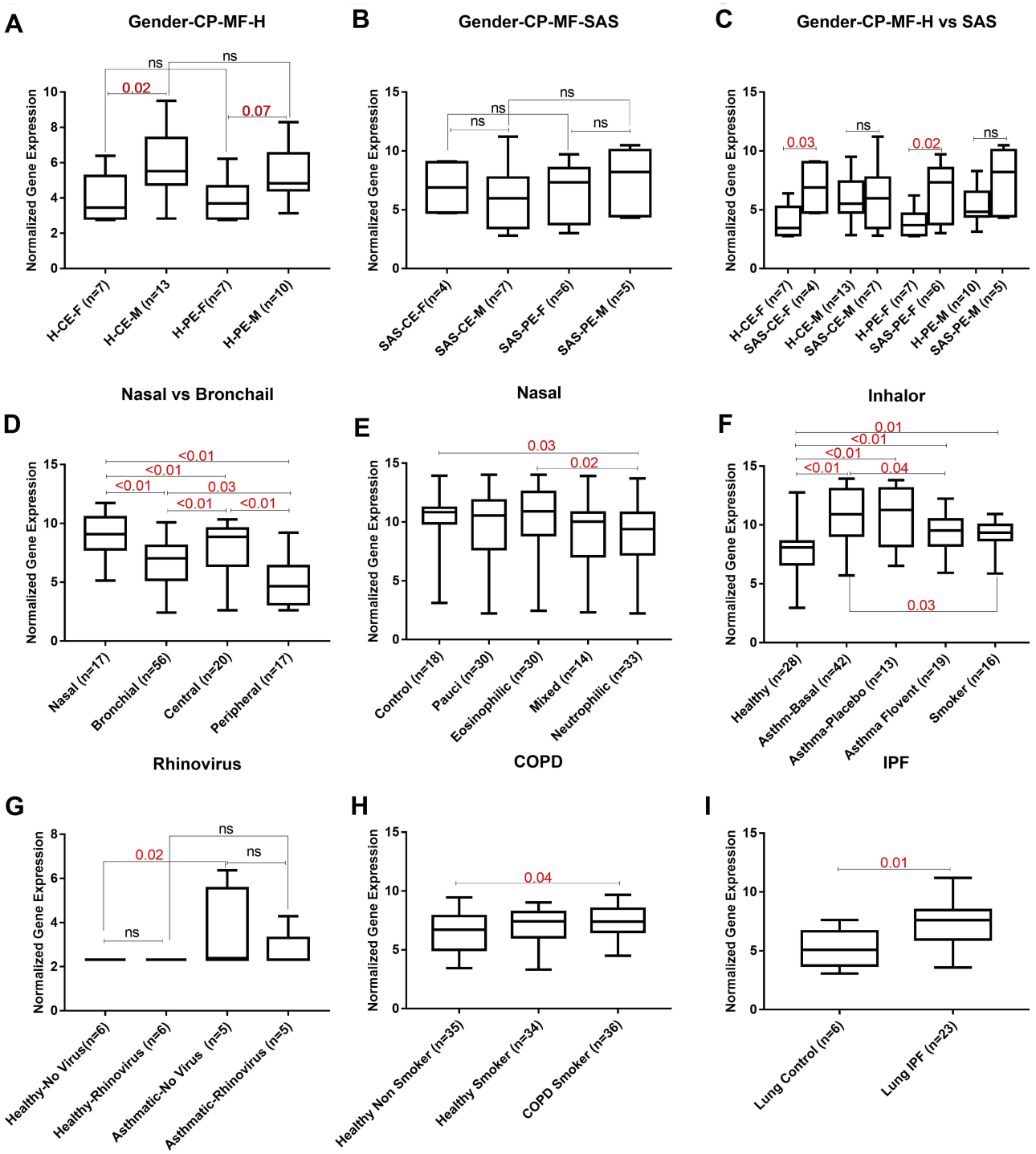

Figure 4 Raw mRNA expression of *POSTN* in the extracted 10 datasets to identify confounding variables that affect *POSTN* gene mRNA expression in healthy "H" and asthmatic "SAS". (**A, B, C**) Gender (female "F" vs male "M") in central "C" vs peripheral "P" airways epithelium "E" (GSE64913), (**D**) Nasal vs Bronchial (GSE4302, GSE64913, GSE13396 and GSE41861), (**E**) Different asthma phenotypes in nasal samples (GSE41862), (**F**) Flovent inhaler use and smoking (GSE4302), (**G**) Rhinovirus infection (GSE13396), (**H**) COPD and smoking (GSE30063) and (**I**) IPF (GSE21369). The Kruskal–Wallis with corrected Dunn's post hoc nonparametric test was performed where $p<0.05$ was considered significant.

Besides, smoking alone showed a significant effect on *POSTN* expression as smokers showed higher expression compared to healthy ($p=0.01$) but lower than asthmatic ($p=0.03$), as shown in Figure 4F. *POSTN* gene mRNA expression was upregulated in the asthmatic bronchial epithelium ($p<0.01$, Figure 4E) but not in asthmatic blood samples compared to healthy(figure not shown).

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Plasma and Saliva POSTN Levels are Elevated in Asthma

Our bioinformatics assessment suggested that *POSTN* can serve as a candidate for a good biomarker if the sample type and the confounding patient's characteristics are taken into consideration. Intriguingly, the subsequent hypothesis was whether the level of POSTN in saliva or plasma of asthmatic patients can be used to differentiate between levels of asthma severity. Hence, we investigated the levels of POSTN in saliva and plasma using a cohort composed of 32 asthmatic patients and 12 non-asthmatic volunteer subjects. As demonstrated in Figure 5A, asthmatic patients (non-severe and severe) showed a significant elevation in the levels of plasma POSTN compared to the healthy group (p=0.04 & p=0.02 respectively). Moreover, POSTN plasma levels were higher (not significant) in the severe asthmatic patients compared to non-severe asthmatics. On the other hand, saliva POSTN level was significantly higher in non-severe asthmatic patients when compared to severe asthmatics (p=0.02, Figure 5C) or healthy controls (p=0.04).

Asthma Treatment and BMI Affect the Level of Plasma and Saliva POSTN

Upon further categorizing the patients: non-severe asthmatic subjects were divided into mild and moderate, while the severe asthmatics were divided into those on Xolair (omalizumab) treatment and those who are not using Xolair. As shown in Figure 5B, mild asthmatics have significantly higher plasma POSTN than healthy controls (p=0.03). Moreover, severe asthmatics who are not using Xolair specifically had higher POSTN plasma levels compared to healthy controls (p=0.02). Regarding saliva POSTN, mild and moderate asthmatic patients showed higher saliva POSTN level than severe asthmatic who are not receiving Xolair (p=0.02 and p=0.03, respectively), as shown in Figure 5D.

Among the clinical and laboratory data of patients, only the use of Symbicort correlated positively with the plasma level of POSTN (r=0.39, p=0.02, Figure 6A). Plasma POSTN level was significantly higher in patients who used Symbicort (49.4±15.51 pg/mL) compared to those who did not use it (37±13.84 pg/mL), as shown in Figure 6B. This confirms our initial assumption that POSTN is an asthma specific biomarker and is elevated in response to treatment. Another interesting finding is the effect of BMI on POSTN plasma level. Class I obese (BMI 30.0–34.9) showed higher POSTN plasma level compared to the overweight class (BMI 25.0–29.9, p=0.04), and compared to class II and class III obese (BMI≥35.0, p=0.02) participants, as shown in Figure 6C and D.

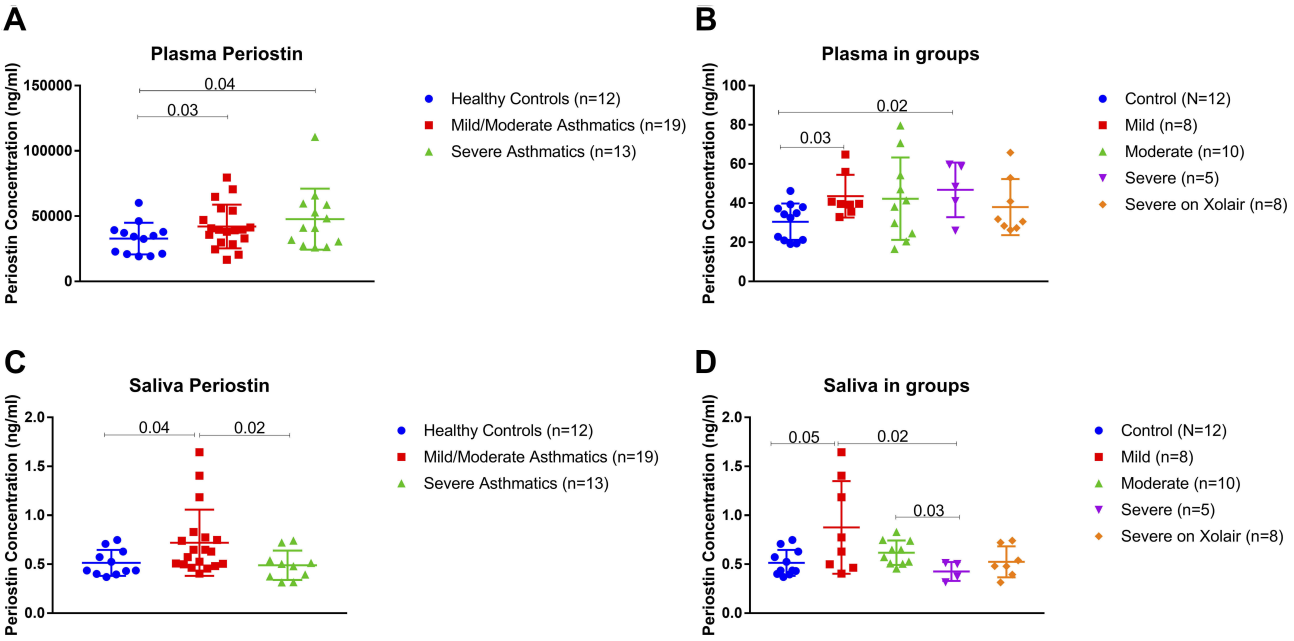

Figure 5 ELISA Quantification of Plasma and Saliva POSTN protein level using a locally recruited cohort of asthmatics patients compared to healthy controls. Plasma (**A-B**) and Saliva (**C-D**) POSTN protein levels in healthy controls versus nonsevere asthmatics (mild and moderate) and severe asthmatics (on Xolair and without Xolair). The Kruskal–Wallis with uncorrected Dunn's post hoc nonparametric test was performed where p<0.05 was considered significant.

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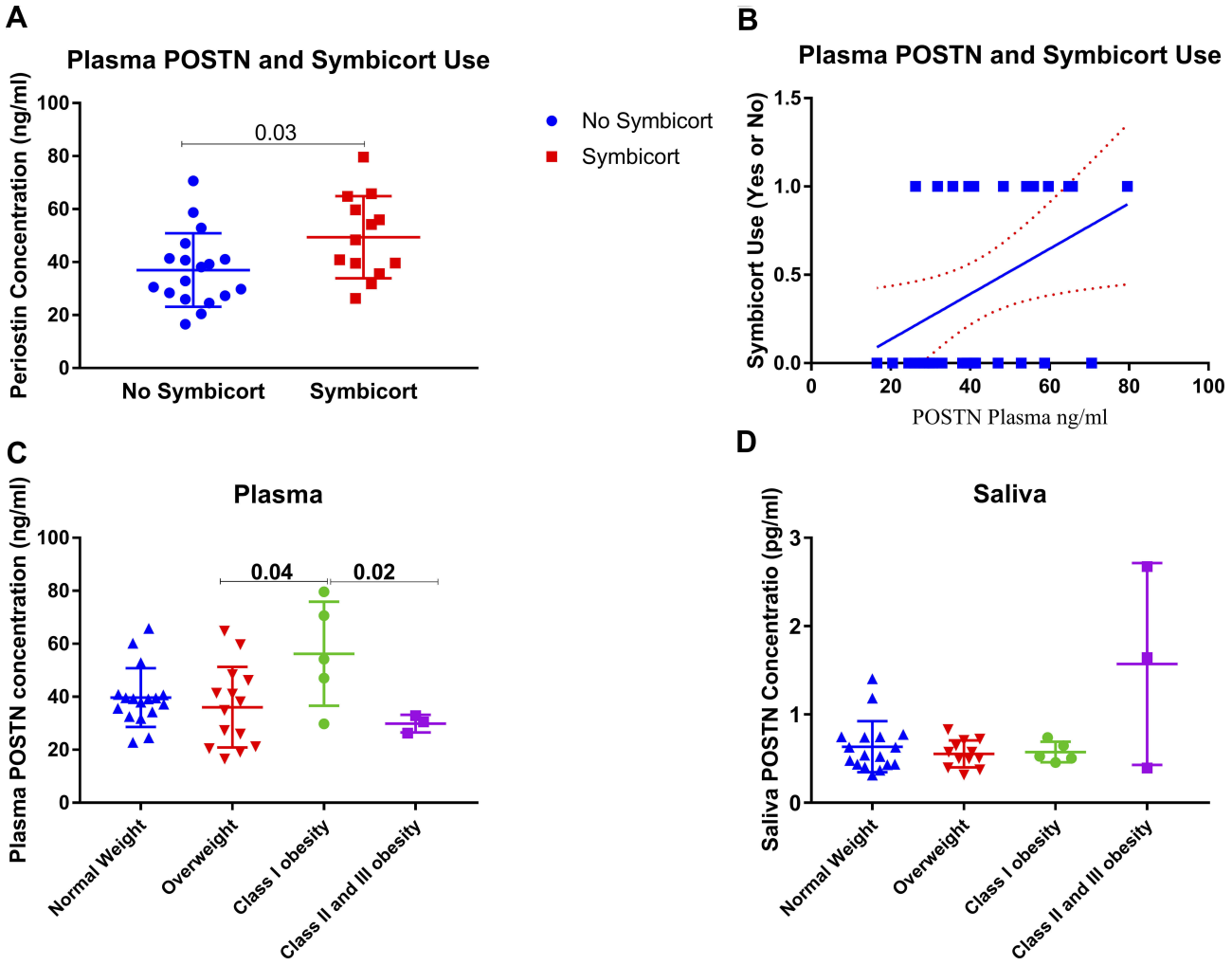

Figure 6 ELISA Quantification of Plasma and Saliva POSTN protein level using ELISA in a locally recruited cohort of asthmatics patients compared to healthy controls. (**A-B**) POSTN level in patients using Symbicort compared to those who are not (**C-D**) plasma and saliva POSTN level in different categories of participants according to their BMI. The Kruskal–Wallis with uncorrected Dunn's post hoc nonparametric test was performed where $p<0.05$ was considered significant.

These results suggest that both plasma and saliva POSTN levels can be used as early markers for asthmatic patients. Furthermore, patients' characteristics and treatment history should be carefully reviewed before any meaningful interpretation of POSTN in level in the clinical practice. Moreover, combining the use of saliva and plasma POSTN can aid in early diagnosis of asthma as well as in the prediction of asthma phenotype and distinction between mild and severe asthma categories.

Discussion

There is an imminent need to discover new biomarkers that can discriminate and classify different asthmatic phenotypes, predict the clinical behavior and therapeutic response as well as aid in the identification of new therapeutic targets.[8,9] Those markers need to be less invasive and more technically convenient in comparison to the currently available ones.[10] Despite that necessity, only a few markers were shown to have potential roles in the clinical practice.[11] However, some of these successful biomarkers showed significant limitations in different populations and settings. This can be accredited to the absence of a unified system of patients' classification and lack of complete understanding of confounding comorbidities, co-existing conditions, sampling time, and type besides the treatment history of asthmatic patients involved in the assessment.[12] For example, atopic status, smoking, and use of inhaled corticosteroids (ICS) are known confounders for the interpretation of asthma biomarkers.[13]

In this study, we used bioinformatics analysis of publicly available transcriptomics data to identify asthma

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specific genes and pathways that are differentially expressed between healthy controls and asthmatic patients' bronchial epithelium. Our approach confirmed that *POSTN* is consistently differentially expressed in a large number of patients across different datasets using bronchial epithelium transcriptomic data. *POSTN* is part of the asthma specific type 2 cytokine signature as it is a downstream molecule of interleukin (IL)-13, and plays a vital role in its pathogenesis.[19] IL-13 induces the production of this matricellular protein from airway epithelial cells and lung fibroblasts in response to injury. IL-13 serum levels can serve as a biomarker for eosinophilic airway inflammation[20] and is associated with asthma disease severity.[21] The exact molecular mechanisms of POSTN involvement in human asthma has not been fully understood.[22] Nevertheless, POSTN was shown to be involved in airway remodeling, subepithelial fibrosis, and mucus production[19] by binding to other extracellular matrix (ECM) proteins.[23] It was reported that POSTN uses its FAS1 domains to bind to cells and its N and C terminals to bind to those ECM proteins, thus acting as a pro-survival protein in many cellular contexts.[24]

On the other hand, high amounts of POSTN could interact with TNFα or IL-1α in fibroblasts to stimulate NF-κB that could result in lung fibrosis.[25] It is well known that high serum POSTN can be a predictive marker for effective anti-IL-13 antibody treatment in asthmatic patients[26] and a prognostic marker for increased risk of asthma exacerbations and progressive decline in lung function.[6] However, whether increased POSTN expression has protective or deleterious effects in asthma, is still a matter of debate. It has been previously reported that POSTN deficient mice challenged with inhaled aeroallergen showed an exaggerated serum IgE and bronchial hyperresponsiveness that might be due to the POSTN effect on TGF-β-induced T regulatory cell differentiation.[25] Moreover, POSTN deficiency was shown to enhance polyps like lesion formation and mast cell infiltration.[27]

Technical challenges that hinder the use of POSTN include post-translational modifications as well as the existence of multiple differentially spliced isoforms in various proportions in diverse tissues and disease states.[28] Proper clinical utility of serum POSTN levels depends on a better understanding of the factors that may affect its level.[29] For instance, baseline POSTN levels were high in children and hence might mask any changes due to its local release within the airways leading to inconsistent findings in children.[9] Therefore, we explored publicly available datasets to identify possible confounders affecting *POSTN* gene expression and hence may limit the interpretation of its level in the clinical settings. Our analysis showed that POSTN gene mRNA expression was upregulated in the asthmatic, smoker COPD and IPF bronchial epithelium when compared to matching healthy controls (Figure 4). This goes in line with the fact that bronchial asthma and COPD possess many similarities that sometimes confuse therapists in their diagnosis and management.[30] Also, this suggests that high *POSTN* mRNA expression cannot rule out any other respiratory diseases if taken alone, and its interpretation should be accompanied by other clinical data and laboratory results. Another suspected factor is human rhinovirus infection (HRV), which is known to cause loss of control in asthmatic symptoms thus leading to emergency room visits and augmentation in asthma management.[31] Such common respiratory viruses are known to induce local POSTN levels to shift immune responses toward Th2 in children[32] especially during a rhinovirus-induced asthma exacerbation.[33] In our analysis using adult bronchial epithelium, there was no effect of HRV on *POSTN* expression. This could indicate that increased *POSTN* level in asthma does not indicate an acute exacerbation induced by HRV.

One of the interesting results in our analysis is the observed effect of fluticasone propionate (Flovent) inhaler on *POSTN* expression. Flovent was found to significantly downregulate *POSTN* mRNA expression in comparison to healthy controls as well as asthmatic patients before initiation of therapy. This supports previous data documenting that serum POSTN levels respond partially to ICS as reflected by a reduction in airway inflammation and wall thickening in asthma.[34] Moreover, Flovent was shown to inhibit POSTN production and its activation of Src/AKT/mTOR pathway.[35] Therefore, this finding confirmed that *POSTN* expression can be used to monitor the response or patient compliance with Flovent treatment. On the other hand, the use of Symbicort correlated positively with the plasma level of POSTN (Figure 5). Compared to Flovent, Symbicort is more effective than a high dose of fluticasone propionate in improving lung function of moderate persistent asthma.[36] The high level of POSTN observed in the group using Symbicort might indicate that this subcategory is not well-controlled and needed Symbicort to control the symptoms. Another possible explanation could be that *POSTN* mRNA is affected specifically with the

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fluticasone present in the Flovent but not with the budesonide in the Symbicort therapy.

It was previously mentioned that the potential of POSTN in predicting a beneficial response to therapies, needs further investigation, and careful interpretation in smoking asthmatics.[37] In our analysis, smoking showed a significant effect on upregulating *POSTN* expression compared to healthy non-smoker individuals (Figure 4). Considering the gender effect on *POSTN* expression, it was found to be higher in males compared to females in healthy bronchial epithelium. At the same time, asthmatic bronchial epithelium expressed higher *POSTN* mRNA in comparison to healthy controls only in females (not males). This goes in line with the previous report in the Asian population where POSTN levels were found to be sex-dependent where females had higher POSTN levels.[29] However, this finding is contrary to a previous study stating that a patient's sex should not be taken into account while assessing serum POSTN.[38] This is a critical point in the recruitment of controls and asthma patients in order to understand and appreciate the male to female ratio in the studied groups.

Our analysis showed that *POSTN* has high similarity scores to 14 genes from the identified DEG between severe asthmatics and healthy controls. This similarity might indicate that *POSTN* can reflect the expression of these genes and hence be a representative biomarker of their underlying interactive pathways. Unsurprisingly, these identified genes were found to be enriched in the pathways related to the development and maintenance of epithelial cells, their response to wounding and immune-related pathways. Regarding their tissue distribution, 13–15 of the identified genes were observed to be related to lung, skin, and gut: highly rich collagen-containing structures that are known to tolerate mechanical stressors. This goes in line with previous literature indicating that POSTN is preferentially expressed in the periosteum as well as other connective tissues that tolerate mechanical stress such as periodontal ligament, heart valves, and tendons.[24] Cumulatively, this can suggest that different tissue sampling other than the respiratory system might reflect expression changes that occur in asthma and can be an alternative for bronchial epithelium in the clinical setting.

In order to understand if there is any similarity in *POSTN* expression between the nasal and upper lung airways, large datasets were explored. Upper airways nasal epithelium showed higher *POSTN* expression compared to the lower bronchial epithelium. In other words, the more peripheral the airways are, the lower is the *POSTN* expression. Moreover, neutrophilic asthma phenotype showed lower nasal *POSTN* expression compared to healthy controls and eosinophilic phenotype. Thus, our findings confirm previous studies where nasal cells expressed higher *POSTN* than bronchial, highlighting that nasal epithelial cells would be a more suitable surrogate for bronchial cells.[39]

In this study, we evaluated serum POSTN using a locally recruited patient cohort consisting of 32 asthmatics and 12 healthy controls. Asthmatic patients (non-severe and severe) showed a significantly higher level of plasma POSTN than healthy controls. These findings confirm that POSTN is upregulated in asthma but cannot differentiate between non-severe and severe asthmatics (Figure 5). Regarding non-severe asthmatics, the mild patients showed a significantly higher level of plasma POSTN compared to healthy controls while there was no significant difference between moderate asthmatics and healthy controls. This could be due to the difference in low-dose inhaled corticosteroids (ICSs) intake in each category since mild asthmatics receive low doses of ICSs while moderate ones receive higher doses of ICSs. These data confirm our bioinformatics results and previous reports where ICSs might suppress POSTN-induced asthmatic inflammation by reducing its serum level.[40] On the other hand, only severe asthmatics who are not on Omalizumab (Xolair) showed significantly higher levels of plasma POSTN compared to healthy controls but severe asthmatics on Xolair did not show a significant difference compared to healthy controls. Xolair is a humanized anti-IgE monoclonal antibody that was shown to decrease the level of POSTN in patients with severe allergic asthma.[41]

Additionally, POSTN serum level was suggested as a biomarker for patient selection and predictor of Xolair response in previous studies.[42]

Another interesting finding in our study is the effect of BMI on POSTN plasma level with no effect on the saliva POSTN level. Class I obese participants showed higher POSTN plasma levels in comparison to overweight and class II-III obese patients, who exhibited the lowest POSTN plasma level. There was a controversial view in the literature where some studies have shown that BMI is in a negative association with serum POSTN in both healthy and asthmatic subjects,[43,44] while others showed that there is no significant correlation between BMI and serum POSTN level.[45]

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Due to the direct anatomic relation between the mouth and the upper airways and their similarity in cytokine composition with the lower airways in asthma, salivary analysis of POSTN could offer noninvasive rapid diagnostic aid in asthma control deterioration.[46] Based on that, we evaluated the diagnostic value of saliva compared to the routine serum POSTN using the same locally recruited patient cohort. Only the mild asthmatic subcategory of non-severe asthmatics showed a higher level of saliva POSTN. On the contrary, severe asthmatic without Xolair therapy showed lower levels of saliva POSTN in comparison to moderate asthmatics. The decrease in POSTN level observed in severe asthmatics could be possibly explained by the local effect of steroid therapy that is frequently taken by moderate and severe asthmatics. The use of saliva POSTN as an inflammatory marker was previously investigated and promoted in periodontal disease,[47,48] where POSTN levels were found to be significantly reduced in its experimental model.[49] Up to our knowledge, this is the first study examining the level of POSTN in the saliva of asthmatic patients. Based on our findings, saliva POSTN level can be possibly used in discriminating the mild early stages of asthma from the healthy state. It would also be used as an early simple cost-effective screening tool to identify early asthma diagnoses to prevent complications.

Conclusion

Our results confirmed that *POSTN* is consistently upregulated in bronchial epithelium of asthmatic compared to healthy ones as well as in other closely related respiratory diseases (COPD and IPF). Its mRNA expression is affected by gender, sample anatomical site and type, steroid therapy, and smoking. Both plasma and saliva POSTN levels were higher in non-severe asthmatics compared to healthy controls and can be used as an early biomarker for asthmatic patients. Saliva POSTN level was more sensitive than plasma POSTN in differentiating between severe and non-severe asthmatics. Finally, patients' characteristics such as BMI and the use of inhaled steroids/biologicals treatment should be carefully reviewed before any meaningful interpretation of POSTN level in the clinical practice.

Disclosure

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