iTRAQ-based proteomic analysis reveals potential serum biomarkers of allergic and non-allergic asthma.

Short title:

Serum biomarkers of allergic and non-allergic asthma

Authors

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42

43 **ABSTRACT**

44 **Background-** Asthma is heterogeneous disease with different phenotypes, endotypes,
45 and severities. Definition of these subgroups requires the identification of biomarkers in
46 biological samples, and serum proteomics is a useful and minimally invasive method for
47 this purpose. Therefore, the aim of this study was to detect serum proteins whose
48 abundance is distinctively associated with different asthma phenotypes (allergic vs. non-
49 allergic) or severities.

50 **Methods-** For each group of donors (32 healthy controls, 43 allergic rhinitis patients, and
51 192 asthmatics with different phenotypes and severities), we generated two pools of sera
52 that were analysed by a shotgun MS approach based on combinatorial peptide ligand
53 libraries and iTRAQ-LC-MS/MS.

54 **Results-** MS analyses identified 18 proteins with a differential abundance.
55 Functional/network study of these proteins identified key processes for asthma
56 pathogenesis, such as complement activation, extracellular matrix organization, platelet
57 activation and degranulation, or post-translational protein phosphorylation. Furthermore,
58 our results highlighted an enrichment of the "*Regulation of Insulin-like Growth Factor
59 (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)*"
60 route in allergic asthma and the lectin pathway of complement activation in non-allergic
61 asthma. Thus, several proteins (e.g., IGFALS, HSPG2, FCN2 or MASP1) displayed a
62 differential abundance between the different groups of donors. Particularly, our results
63 revealed IGFALS as a useful biomarker for moderate-severe allergic asthma.

64 **Conclusion-** Our data suggest a set of serum biomarkers, especially IGFALS, capable
65 of differentiating allergic from non-allergic asthma. These proteins reveal different
66 pathophysiological mechanisms and may be useful in the future for diagnosis, prognosis,
67 or targeted-therapy purposes.

68

69 **KEYWORDS**

70 Allergic asthma, asthma biomarker, IGF-ALS, non-allergic asthma, serum proteomics.

71

72

73 **INTRODUCTION**

74 Asthma is a heterogeneous disease with two major clinical phenotypes [1, 2], allergic
75 asthma (AA) and non-allergic asthma (NAA), which in turn are composed of several
76 endotypes based on the underlying molecular mechanisms [3]. The most prevalent
77 phenotype is AA, which typically develops in childhood and is less severe than NAA [2].
78 AA is driven by type-2 helper T-cells (TH2) and eosinophilic airway inflammation, which
79 explains the predominance of certain TH2 markers ($T2^{high}$ phenotype): IL-5, IL-13,
80 exhaled nitric oxide fraction/FENO, blood/sputum eosinophilia, and serum periostin [4].

81 The airway epithelium has also a key role in AA through the release of IL-25, IL-33, and
82 TSLP, a set of cytokines with a stimulatory role on dendritic cells, basophils, and innate
83 type 2 lymphocytes (ILC2) [5]. Subsequently, ILC2 cells boost the differentiation of TH2
84 and IgE-producing B cells through the release of IL-4 and IL-13 [5]. Allergen-specific IgE
85 has also a central role in AA via activation of mast cells [6]. AA patients may present
86 eczema or food and drug allergy as comorbidities [3], but about 90% suffer from allergic
87 rhinitis (AR). Therefore, these two pathologies could share similar immunopathogenic
88 mechanisms and biomarkers [7].

89 Alternatively, 10-50% of adult patients with asthma display a less well-known
90 NAA phenotype [1]. This is associated with a negative skin prick test, a severe-persistent
91 disease, the absence of a TH2-signature, and poorer prognosis/response to anti-
92 inflammatory treatment [1]. This asthma phenotype shows a higher prevalence amongst
93 females and a later age of onset [1]. NAA is a more heterogeneous phenotype, including
94 diverse endotypes (e.g., aspirin-exacerbated respiratory disease, obesity-associated
95 asthma, occupational asthma) and different cells involved (e.g., TH17, neutrophils) [8].
96 However, the underlying immunopathological mechanisms in this $T2^{low}$ phenotype are
97 not well understood.

98 From the above mentioned, it seems necessary to perform new studies aimed to
99 search for new biomarkers in asthma. These bioindicators could: a) reveal novel
100 pathophysiological mechanisms; b) discriminate between different asthma
101 phenotypes/endotypes; c) turn into novel targets for treatment. In this way, Clinical
102 Proteomics has paved the way for the discovery of potential diagnostic/prognostic
103 biomarkers in different diseases. Indeed, it has been successfully applied to
104 plasma/serum samples in asthma [9-11], even though there are a very limited number of
105 studies using high-throughput liquid chromatography coupled online to tandem mass
106 spectrometry (LC-MS/MS) [12]. In addition, to our knowledge, none of them were
107 designed to explore the differences in the serum proteome from allergic rhinitis and
108 allergic/non-allergic asthmatic subjects. For this reason, the goal of the present study
109 was double: a) to develop a "shotgun"/"bottom-up" methodology for the quantitative
110 analysis of the medium-low abundance serum proteome; b) to perform a discovery/"non-
111 targeted" proteomic analysis to detect candidate biomarkers associated with the
112 presence of allergic rhinitis or different asthma phenotypes (allergic/non-allergic) or
113 severities (moderate-severe and intermittent-mild), which could also provide new
114 insights into asthma pathogenesis and pathophysiology, as well as improve future
115 therapeutic interventions.

MATERIALS AND METHODS

Please see Supplementary Information for further details.

Donors

A set of 267 adult subjects were recruited at the University Hospital of Santiago de Compostela between 2014 and 2016 and divided into 6 study groups: moderate-severe allergic asthma (MSAA; N=49), intermittent-mild allergic asthma (IMAA; N=53), allergic rhinitis (AR; N=43), moderate-severe non-allergic asthma (MSNAA; N=43), intermittent-mild non-allergic asthma (IMNAA; N=47), and healthy controls (HC; N=32). Asthmatic patients had a confirmed diagnosis of asthma for at least one year (Global Strategy for Asthma Management and Prevention criteria, GINA 2012; https://ginasthma.org/wp-content/uploads/2019/01/2012-GINA.pdf) and were in a stable phase (>4 weeks). A positive bronchodilator test (>12% of FEV1 change after salbutamol) or methacholine challenge were used to confirm asthma diagnosis. Rhinitis was defined as an inflammation of the lining of the nose characterized by nasal symptoms including anterior or posterior rhinorrhoea, sneezing, nasal blockage, and/or itching of the nose. These symptoms occur during two or more consecutive days for more than 1 hour on most days [13]. Skin prick test (SPT) or allergen-specific IgE against the common allergens (Dermatophagoides *pteronyssinus*, Dermatophagoides *farina*e, Lepidoglyphus *destructor*, Tyrophagus *putrescentiae,* Phelum *pratense*, Betula *verrucosa*, Olea *europae*, Canis *familiaris*, Felis *domesticus*, Parietaria *judaica*, Artemisa *vulgaris*, Alternaria *alternata*, and Aspergillus *fumigatus*) in subjects who cannot undergo SPT, were employed to verify the allergic phenotype. Specific IgE levels >0.35 kU_A/L were considered as indicative of sensitization [14]. Samples from the subjects sensitized to pollen were taken outside the relevant pollen season. Haematology, biochemistry, spirometry, and skin sensitization studies were also carried out. The research protocol was approved by the Ethics Committee of Clinical Research of Galicia (2011/001), and all the participants signed an informed consent.

Serum samples preparation.

Two pools of serum samples were created in each group of donors (pool A and pool B). Besides, a reference sample (pooled sample; PS) consisting of all study serum samples was prepared. Then, due to the high affinity of combinatorial peptide ligand libraries (CPLLs) for apolipoproteins, sera delipidation was carried out by means of a LRA^{TM} resin (Lipid Removal Agent, Sigma-Aldrich); to this end, a concentration of 80 mg/mL of resin was selected based on the reduction of apoAI in 1-DE gels (Supplementary figure 1). Afterwards, serum samples were enriched in low abundance proteins by reduction of the dynamic range of protein concentration with CPLLs (ProteoMiner™ Protein Enrichment Kit, Bio-Rad). Then, protein samples were cleaned from interfering substances by means of the 2-D Cleanup Kit (GE Healthcare), reduced ((tris(2-carboxyethyl)phosphine; TCEP), alkylated (methyl methanethiosulfonate; MMTS), and trypsinized. Finally, peptides were labelled by means of iTRAQ® Reagent-8PLEX Multiplex Kit (Sigma-Aldrich) and combined in one single sample (Pools A from each group of donors + PS; Pools B from each group of donors + PS).

158 **nanoLC/MS-MS identification and quantification of peptides**

159 Peptide samples were reconstituted in 0.1% (v/v) formic acid in ultrapure dH_2O and
160 desalted by Zip-Tips C18 (Millipore). Then, samples were analysed by LC-MS/MS by
161 using a Proxeon EASY-nLC 1000 UHPLC (Thermo Fisher Scientific) coupled online to
162 an LTQ-Orbitrap ELITE (Thermo Fisher Scientific). HCD-fragmentation was performed.
163 Three technical replicates were analysed on each biological replicate (Pools A + PS;
164 Pools B + PS).

165 **MS data analysis**

166 Proteome Discoverer (version 2.1.1.21) was used for protein identification and iTRAQ
167 quantification. HCD spectra were analysed using Sequest HT with Percolator validation.
168 The intensities acquired in MS/MS were globally normalized on protein median. Then,
169 all reporter intensities were normalized by the reporter intensity of the PS (121). Different
170 fold change criteria (down-regulation, < 0.77; up-regulation, ≥1.3) in iTRAQ ratios, as
171 well as a q-value <0.05, were used to identify differentially abundant proteins between
172 the groups of study.

173 **Validation of biomarkers by ELISA**

174 IGFALS was selected for further validation by ELISA. For this aim, the Immunotag™
175 Human IGFALS (Insulin-Like Growth Factor Binding Protein, Acid Labile Subunit) ELISA
176 from G-Biosciences was used. Protocol was performed according to the manufacturer´s
177 guidelines.

178 **Statistical and bioinformatic analysis of the data**

179 Multiple t-test analyses with two-stage linear step-up procedure of Benjamini, Krieger
180 and Yekutieli to determine False Discovery Rate (FDR: $q < 0.05$) were used for the
181 detection of differentially abundant proteins across the different groups (biomarker
182 discovery study). To analyse the changes in the clinical and haematological variables
183 between the different groups, as well as for ELISA results, Kruskal–Wallis one-way
184 analysis of variance followed by Dunn's tests were performed. All these analyses, as well
185 as the receiver operating characteristic (ROC) analyses, were carried out with GraphPad
186 Prism.

187 Gene Ontology (GO) annotation, GO terms overrepresentation test, and Reactome
188 pathway overrepresentation analyses against the plasma proteome database (Version
189 2015-02-02) were performed in the PANTHER Classification System (version 13.1).
190 Protein-protein interaction analyses were also performed by means of the Search Tool
191 for the Retrieval of Interacting Genes (STRING v11; http://string-db.org/).

192

193

RESULTS

Clinical characteristics of the study patients

This work includes 267 adult patients with different asthma phenotypes (AA and NAA) and severities (Intermittent-mild and moderate-severe), a group with allergic rhinitis (AR) and another group of healthy controls (HC). The characteristics of the different study groups are outlined in Table 1. As expected, patients with moderate-severe asthma had a decrease in forced expiratory volume in one second (FEV1%) and FEV1/forced vital capacity (FVC) compared with patients with intermittent-mild asthma, although the reduction of FEV1% is only significant in the case of the NAA (Table 1). In addition, both FEV1% and FEV1/FVC ratio were also decreased in moderate-severe asthmatic patients with respect to AR (Table 1). AA was also characterized by higher levels of IgE than the remaining groups, except for AR patients (Table 1). On the other hand, BMI and erythrocyte sedimentation rate (ESR) measures were slightly higher in NAA patients compared to AR (Table 1). Leukocyte count was also performed, showing an increase in the number of circulating eosinophils in AA and moderate-severe NAA patients compared to HC, and above AR in the case of moderate-severe AA (Table 1). Other biochemical or haematological parameters studied remained unchanged (Table 1).

Qualitative data analysis and classification of detected proteins according to GO terms.

Serum samples from each group (MSAA, IMAA, AR, MSNAA, IMNAA, and HC) were randomly split into 2 pooled subgroups (Pool A and B; i.e., 2 “biological replicates” per group; see Supplementary tables 1 and 2 for characteristics), which were analysed three times (3 technical replicates). Candidate biomarkers for different phenotypes and severities were identified in serum by using a high-throughput mass-spectrometry (MS) approach, which combined lipid removal with LRA resin to facilitate the identification of proteins other than apolipoproteins, enrichment of low abundance proteins by combinatorial peptide ligand libraries (CPLL; ProteoMiner™), and iTRAQ-LC-MS/MS analysis. The experimental design is shown in Figure 1.

We identified a total of 9157 peptide-spectrum matches, 1403 peptides, and 217 proteins from all study groups. All the proteins were detected in each of the seven groups of the present study at least in one out of three technical replicates from each biological replicate. Classification by biological process (GO-Slim BP) indicated that 4 categories, including cellular and metabolic processes, immune system process, and response to stimulus, represented more than 60% of the proteome detected (Figure 2a). Indeed, there was an enrichment of processes related to the immune system compared to the plasma proteome, such as complement activation, B cell-mediated immunity, or defence response to bacteria; all of them play a key role in asthma pathophysiology (Table 2; biological process). As expected from a serum sample, classification by cellular component (GO-Slim CC) indicated that many proteins still belonged to the extracellular space (47.5%) or macromolecular complexes (19.1%) (Figure 2b); the enrichment analysis also agreed with these results (Table 2; cellular component). Finally, classification by molecular function (GO-Slim MF) indicated that >80% of proteins

displayed binding (44.3%) or catalytic activities (40.2%) (Figure 2c); the GO terms overrepresentation test yielded the same results (Table 2; molecular function).

Quantitative data analysis of proteins identified by shotgun proteomics

iTRAQ reporter intensity values for each group were normalized by the iTRAQ reporter intensity of the pooled sample. Then, multiple t-test analyses with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to determine False Discovery Rate (FDR < 0.05) were used for the detection of differentially abundant proteins across the different groups (Fold change >1.3, q < 0.05). Only proteins identified in more than two "technical replicates" for each "biological duplicate" were used for quantification purposes (N = 145). Eighteen out of 145 proteins identified showed significant changes between the different groups of donors (Supplementary Table 3). In the following paragraphs we report the most significant changes in the proteome of patients with MSAA, AR or MSNAA compared to other donor groups.

Proteins with changes in MSAA compared to other study groups (HC, IMAA, MSNAA) were shown in Figure 3. Seven proteins (AMBP, IGFALS, PROS1, KNG1, CALU, C4B, and C1R), were found increased in MSAA compared to HC (Supplementary Table 3). Three of them (PROS1, KNG1, and CALU) were proteins implicated in platelet activation and degranulation (HLA-76002, HLA-114608) that display a high level of interrelation (0.900) (Figure 3). The same strong interaction was found for two proteins from the complement cascade, C4B and C1R (Figure 3). On the other hand, nine proteins were found upregulated in MSAA compared to MSNAA (AMBP, NCAM1, AHSG, F2, CALU, C4B, IGFALS, HSPG2, and SOD3) (Figure 3). Interactome analyses of these proteins indicate a high interaction score (0.900) for five of these proteins: AMBP, AHSG, F2, CALU, and IGFALS (Figure 3). Interestingly, four of them (IGFALS, AHSG, F2, and CALU) belong to the Reactome pathway "*Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)"* (HLA-381426) (Figure 3). Indeed, PANTHER overrepresentation test against plasma proteome database highlighted a 46.69-fold enrichment of this pathway (FDR, 2.33E-03) in MSAA. Other interesting proteins that increased their levels in MSAA compared to MSNAA included HSPG2 and NCAM1; both proteins are implicated in extracellular matrix organization (HLA-1474244) (Figure 3). We also found an increase of proteins from the Reactome pathways "*Metabolism of proteins*" (HLA-392499), "*Formation of Fibrin Clot"* (HLA-140877), and "*Post-Translational Protein Phosphorylation*" (HLA-8957275) (Figure 3) in MSAA vs. MSNAA. Finally, only one protein, ORM1, was found increased in MSAA compared to IMAA patients (Figure 3).

On the other hand, proteins related to extracellular matrix organization (HSPG2 and NCAM1) (HLA-147-4244) and proteins from the reactome pathway "*Regulation of complement cascade*" (CPN1 and C1R; HLA-977606) were found increased in allergic rhinitis compared to HC (Figure 4). In addition, certain proteins linked to platelet activation and degranulation (HLA-76002; HLA-114608), such as CALU, AHSG, and FGB, were upregulated in allergic rhinitis patients compared to MSNAA (Figure 4; Supplementary Table 3), something similar to what happens in MSAA vs. MSNAA patients (Figure 3). Interestingly, only one out of nine proteins displayed a reduced abundance in allergic rhinitis compared to MSNAA patients; that protein was a

component of the lectin pathway of complement activation called mannan-binding lectin serine protease 1 (MASP1).

Interestingly, only two proteins were upregulated in MSNAA compared to allergic rhinitis patients and HC: the above-mentioned MASP1 serine-protease and a pattern-recognition molecule (ficolin-2 / FCN2), respectively (Figure 5). Classification of those proteins by biological processes indicated that both proteins were related to lectin pathway of complement activation (GO0001867). On the contrary, most proteins were found downregulated in MSNAA compared to allergic patients (both allergic rhinitis and allergic asthma). Between them, proteins related to extracellular matrix organization (HSPG2, NCAM1, or FGB; HLA-1474244), proteins associated to platelet activation/degranulation (CALU, AHSG, F2, or FGB; HLA-76002; HLA-114608) or, as described before, proteins related to the IGF-family (HLA-381426): AHSG, F2, CALU, and especially IGFALS (Figure 5; Supplementary Table 3).

Validation of IGFALS by ELISA

Due to the predominance of proteins related to the IGF pathway (AHSG, F2, and CALU and IGFALS) as possible differentiating biomarkers of allergic asthma from non-allergic asthma, IGFALS was chosen for subsequent validation by ELISA (Figure 6). Serum samples coming from subjects belonging to each study group and with similar age and M/F proportion were included in this confirmatory analysis. Despite the small sample size in this preliminary study (n=13), there was an increase in the IGFALS concentration in MSAA patients compared to the remaining study groups, except for MSNAA patients (dot plot in Figure 6). Moreover, ROC analyses highlighted this single serum biomarker allowed to discriminate MSAA from the remaining groups with a reasonable performance (Figure 6). Therefore, IGFALS could be a useful biomarker in defining severity (moderate-severe) and allergic phenotype in asthma.

DISCUSSION

In the present study, a protocol for the proteomic study of human serum samples has been optimized. In this protocol, lipoproteins were eliminated, the dynamic range of protein abundances reduced by CPLLs, and a qualitative/quantitative analysis performed (iTRAQ-LC-MS/MS). A preliminary study of the serum proteome in different donor groups (MSAA, IMAA, AR, MSNAA, IMNAA, HC) demonstrated an enrichment of proteins that perform their biological function in the extracellular region. These proteins exhibited binding or catalytic activity, and their biological processes were mainly related to metabolism or immune system (e.g., complement activation, B cell immunity, defence against bacteria, or phagocytosis). Therefore, they constitute a subproteome enriched in potential biomarker candidates for asthmatic phenotypes/endotypes. In consonance with this assertion, our study reported a differential abundance of several proteins in the serum proteome of patients with allergic rhinitis or AA/NAA phenotypes. One of them, IGFALS, has been described as a possible biomarker of moderate-severe allergic asthma.

323 The usefulness of the LC-MS/MS approach combined with the use of iTRAQ
324 isobaric labels has been proven in numerous publications [15, 16]. Although several
325 proteomic studies have been performed in the asthma field [9-11], only one has used
326 iTRAQ-LC-MS/MS [17]; moreover, none of them included allergic rhinitis and different
327 asthma phenotypes (AA vs. NAA) and severities in the study groups. In the present
328 study, we have obtained a list of 217 proteins, 18 of them displaying a differential
329 abundance across the different groups of donors. Interestingly, some of those proteins
330 could be relevant as candidate biomarkers for asthma phenotypes diagnosis or
331 assessment of asthma severity. This is the case, for example, of FCN2 and MASP1 for
332 NAA, or HSPG2 and IGFALS for AA.

333 IGFALS belongs to a family of proteins previously linked to asthma pathogenesis.
334 This group of proteins includes IGF1 and IGF2 [18], the first one a key peptide hormone
335 that promotes subepithelial fibrosis, inflammation, hyperresponsiveness, and smooth
336 muscle cell hyperplasia in the airways [18]. Indeed, omalizumab (an anti-IgE antibody)
337 [19], as well as oral glucocorticoids [20], decrease the levels of IGF-I. Both IGF1 and
338 IGF2 exert their biological effects through the binding to IGF1R, but IGF2 can be also
339 recognised by IGF2R/cation-independent mannose-6-phosphate receptor, a high-affinity
340 inhibitory protein that attenuates IGF2 signalling [21]. Strikingly, IGF2R acts as a receptor
341 for soluble CD26/DPP4 [22], a serine peptidase that mitigates the biological functions of
342 cytokines/chemokines and involved in asthma pathogenesis [23]. Therefore, different
343 lines of evidence point to an important role of IGF family in allergic asthma.

344 The IGF system is rounded off by several binding proteins (IGFBPs: 1-6) [24], a
345 novel IGFBP3-specific receptor (IGFBP3R) [25], and IGFALS [24, 26]. Regarding
346 IGFBP3, this protein controls AA inflammation through IGF-dependent and IGF-
347 independent (IGFBPR-mediated) mechanisms that target the HIF/VEGF axis, NF-kB
348 activation, and TGFβ1 and TH2 cytokines production [18]. Veraldi *et al.* also described
349 a role in AA for IGFBP-3 through the promotion of subepithelial fibrosis [27]. IGFBP4, on
350 the other hand, has been found increased in severe asthma and decreased after
351 Omalizumab treatment [28]. To complete the picture collectively formed by IGF1 [18],
352 IGFBP3 [18], and IGFBP4 [28], our results show that IGFALS is elevated in AA,
353 especially moderate-severe forms, but not in NAA. Both IGFBPs and IGFALS appear to
354 influence free-IGF concentration in the extracellular compartment, being determinants of
355 the bioavailability of this hormone [18]. The formation of a ternary complex (IGFALS-
356 IGF1-IGFBP) prevents the extravasation of IGF-1, the IGF-1/IGFBPs proteolysis, and
357 the renal elimination of IGF-1 [29]. Indeed, IGFALS deficiency results in a dramatic
358 decrease in IGF-1, IGF-2, and IGFBP3 levels [29, 30]. Regarding the specific role of
359 IGFALS in asthma, this issue remains unknown, but together with IGFs and IGFBPs this
360 protein could turn into an additional therapeutic target in asthma.

361 Our results reveal several similarities between allergic asthma and allergic
362 rhinitis, especially related to proteins implicated in extracellular matrix organization.
363 Pending on further confirmation, a potential biomarker for atopic disease appears to be
364 HSPG2/perlecan. Apart from our own data, several lines of evidence support this
365 assertion. Thus, HSPG2 is an extracellular matrix molecule constitutively produced by
366 fibrocytes [31] that generates subepithelial fibrosis in the airways [32]. Apart from fibrosis,
367 it has been observed a negative correlation between the levels of HSPG2 and airway
368 hyperresponsiveness [33]. Moreover, fibrocytes from asthmatic patients exposed to TH2

369 (IL-4, IL-13) but not TH17 (IL-17A) cytokines show enhanced expression of HSPG2 and
370 a profibrotic phenotype [34]. Taken together, these results could explain the increase of
371 HSPG2 in MSAA and allergic rhinitis patients.

372 The complement system also appears to have a proinflammatory role in asthma.
373 Interestingly, our preliminary results support that C4B and C1r proteins (classical
374 complement pathway) are upregulated in atopic diseases (AR, AA), whereas MASP1
375 and FCN2 (lectin complement pathway) appear to be also increased in non-allergic
376 asthma. MASP1 participate in the lectin pathway of complement activation, but this
377 serine protease also has substrates that belong to the coagulation cascade [35]; we have
378 found some of them altered in asthma, such as kininogen (KNG1) or F2. Moreover,
379 another substrate of MASP1 on endothelial cells is the protease activation receptor 4
380 (PAR4), whose activation leads to the production of IL-8, a chemotactic molecule for
381 neutrophils [35]. This is relevant, as neutrophils have been related to some non-allergic
382 asthma endotypes [36] and higher disease severity [37]. On the other hand, L-
383 ficolin/Ficolin-2/FCN2 is a lectin that recognizes bacteria, fungi and apoptotic cells and
384 whose evidence relating with asthma is sparse. Only a single observational study
385 reported a reduction of serum FCN2 concentration in children with asthma and/or allergic
386 rhinitis [38], but to our knowledge there are no descriptions of FCN2 levels in NAA.
387 Therefore, our results point out that, depending on the asthma phenotype, different
388 complement activation pathways could be involved: the classic pathway in AA and the
389 lectin pathway in NAA. Therefore, future research on asthma phenotypes/endotypes
390 should include functional measurements of these complement pathways.

391

392 CONCLUSION

393 A shotgun/bottom-up/non-targeted methodology has been developed for the
394 quantitative analysis of the proteome of medium-low abundance in serum samples from
395 patients with allergic rhinitis and various asthmatic phenotypes. This protocol is based
396 on the elimination of lipoproteins by an LRA resin, protein enrichment of medium and low
397 abundance by CPLLs application, labelling with iTRAQ 8plex reagents, and analysis by
398 LC-MS/MS. Despite the technological limitations due to serum pooling and the potential
399 difficulties in detecting differences in protein abundances between the groups of donors
400 due to the use of CPLLs, our approach detected several potential biomarkers of asthma
401 phenotypes or severities. One of them, IGFALS, has been further validated as an
402 indicator of MSAA. Despite these suggestive results, future studies will be necessary to
403 get a better understanding of asthma pathophysiology, to further validate and evaluate
404 the diagnostic performance of these new biomarkers in different phenotypes/endotypes,
405 and to translate this knowledge into the clinic to get a better therapeutic response and
406 prognosis.

407

408 **CONFLICT OF INTEREST STATEMENT**

409 The authors declare no conflict of interest.

410

AUTHOR CONTRIBUTIONS

The experimental part of the study was carried out by JJN-F, LJA-B, and FJS. JJN-F and RMC performed the statistical analyses. JJN-F, FJG-B, PA, and FJS participated in the study design and article writing. FJG-B, MES-J, MJC, and LV-C carried out sample collection and the clinical part of the study. All authors critically reviewed the manuscript and approved the final draft to be published.

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553

TABLES

Table 1. Clinical and demographic characteristics of the study subjects.

	MSAA	IMAA	MSNAA	IMNAA	AR	HC
N	49	53	43	47	43	32
Age (mean (range))	39 (18-68)	36 (20-66)	54 (24-68)	52 (29-72)	35 (18-55)	43 (22-61)
Sex (M/F)	23/26	25/28	13/30	8/39	24/19	15/17
Disease control:						
Yes	30	53	30	45	43	-
No	19	0	13	2	0	-
Baseline treatment:						
ICS-LABA	46	34	39	35	0	-
ICS	1	15	1	3	0	-
OCS	1	0	0	0	0	-
Antileukotrienes	25	18	21	9	10	-
Anticholinergic	13	3	25	2	0	-
Roflumilast	0	0	1	0	0	-
Prednisone	0	0	5	0	0	-
BMI (Kg/m^2)	26.9 (24.1-30.1)	25.3 (23.2-30.3)	29.2 (26.8-33.5)$^{\#}$	27.4 (255.4-30.5)$^{\#}$	25.3 (22.3-27.4)	-
FEV1 (%)	93.0 (81.5-103.0)$^{\#}$	101.0 (93.5-109.0)	75.0 (65.0-94.0)$^{\#\&}$	110.0 (97.0-116.0)	107.0 (99.0-119.0)	-
FEV1/FVC (%)	73.0 (64.1-79.2)$^{\#\&}$	79.1 (72.5-81.8)$^{\#}$	67.2 (59.5-76.6)$^{\#\&}$	76.6 (72.6-80.5)$^{\#}$	83.5 (79.0-87.4)	-
Neutrophils (10^3 cells/µL)	3.77 (2.75-4.50)	3.71 (3.17-4.27)	3.58 (3.03-4.49)	3.51 (2.72-4.13)	3.58 (2.93-4.00)	3.02 (2.28-4.05)
Lymphocytes (10^3cells/µL)	1.98 (1.65-2.66)	1.99 (1.67-2.24)	1.94 (1.56-2.54)	1.95 (1.58-2.25)	2.16 (1.89-2.60)	1.99 (1.53-2.49)
Monocytes (10^3 cells/µL)	0.39 (0.31-0.48)	0.41 (0.31-0.48)	0.42 (0.32-0.52)	0.35 (0.30-0.47)	0.39 (0.31-0.47)	0.39 (0.33-0.49)
Eosinophils (10^3 cells/µL)	0.36 (0.22-0.53)$^{*\#}$	0.26 (0.18-0.42)*	0.30 (0.18-0.45)*	0.24 (0.14-0.37)	0.22 (0.14-0.29)	0.13 (0.09-0.22)
Basophils (10^3 cells/µL)	0.04 (0.03-0.05)	0.04 (0.03-0.05)	0.03 (0.02-0.05)	0.03 (0.02-0.04)	0.03 (0.02-0.05)	0.03 (0.02-0.05)
ESR (1h; mm)	8.0 (4.5-15.0)	7.0 (2.0-14.5)	13.0 (7.0-23.0)$^{\#}$	12.0 (8.0-20.0)$^{\#}$	8.0 (2.0-15.0)	7.5 (2.0-10.0)
IgE (IU/mL)	138 (65-374)$^{*\$}$	163 (64-252)$^{*\$}$	43 (15-96)	22 (8-35)	68 (24-124)	15 (6-55)
IgG (mg/dL)	1060 (940-1150)	1080 (932-1210)	1055 (927-1255)	1020 (871-1160)	1025 (915-1208)	1015 (882-1118)
IgA (mg/dL)	213 (161-270)	243 (176-300)	220 (160-270)	206 (149-260)	187 (133-274)	291 (128-333)
IgM (mg/dL)	98 (70-150)	100 (69-143)	115 (76-160)	107 (73-149)	111 (69-163)	95 (86-114)

AA, allergic asthmatics; HC, healthy controls; NAA, non-allergic asthmatics; AR, allergic rhinitis patients.

Data are presented as median value (IQR1-3), unless otherwise expressed.

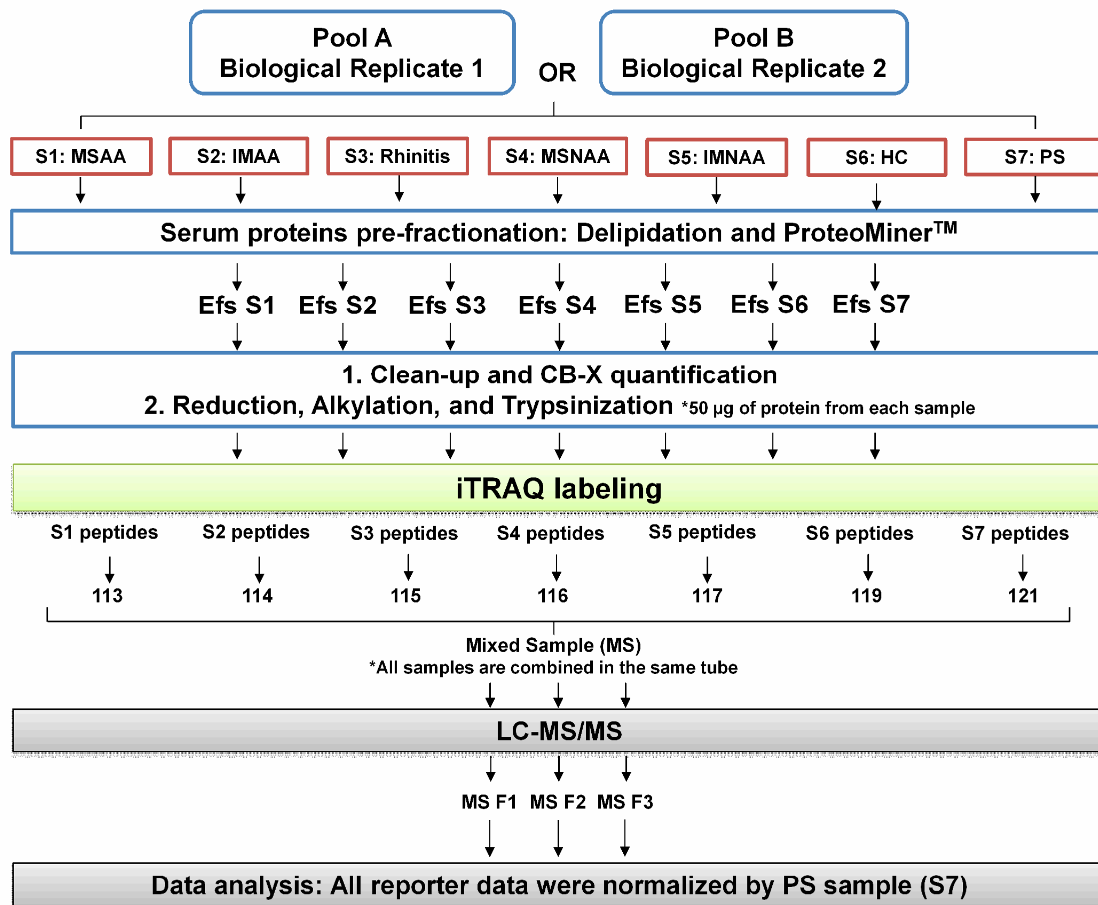
Statistical significance is show: *Disease vs HC; $^{\#}$Asthma vs AR; $^{\$}$AA vs NAA; $^{\&}$Moderate-severe vs Intermittent-mild asthma. Kruskal-Wallis test followed by Dunn´s multiple comparison test. $p < 0.05$

Table 2. GO terms overrepresentation against human plasma proteome.

	Observed	Expected	Fold Enrichment	FDR
PANTHER GO-Slim: Biological Process				
cholesterol metabolic process (GO:0008203)	5	.31	15.93	6.01E-04
complement activation (GO:0006956)	9	.65	13.80	3.15E-06
B cell mediated immunity (GO:0019724)	9	.65	13.80	2.52E-06
cell recognition (GO:0008037)	10	.74	13.58	8.51E-07
defence response to bacterium (GO:0042742)	9	.80	11.29	8.45E-06
immune response (GO:0006955)	31	3.10	9.99	6.52E-19
response to biotic stimulus (GO:0009607)	9	1.01	8.87	4.20E-05
cell-matrix adhesion (GO:0007160)	4	.47	8.49	1.84E-02
phagocytosis (GO:0006909)	10	1.46	6.84	7.90E-05
steroid metabolic process (GO:0008202)	6	.93	6.45	5.87E-03
immune system process (GO:0002376)	35	5.59	6.26	7.17E-16
cell-cell adhesion (GO:0016337)	7	1.29	5.42	5.67E-03
proteolysis (GO:0006508)	13	3.69	3.52	1.82E-03
endocytosis (GO:0006897)	11	3.15	3.49	5.84E-03
response to stress (GO:0006950)	14	5.35	2.62	1.28E-02
protein metabolic process (GO:0019538)	28	12.62	2.22	1.46E-03
cellular process (GO:0009987)	48	66.83	.72	1.77E-02
phosphate-containing compound metabolic process (GO:0006796)	4	13.93	.29	2.51E-02
biosynthetic process (GO:0009058)	3	14.28	.21	6.80E-03
nitrogen compound metabolic process (GO:0006807)	3	20.90	.14	2.71E-05
nucleobase-containing compound metabolic process (GO:0006139)	3	22.98	.13	4.03E-06
PANTHER GO-Slim: Cellular Component				
immunoglobulin complex (GO:0019814)	10	.69	14.53	6.30E-08
extracellular space (GO:0005615)	41	4.66	8.80	8.55E-25
extracellular region (GO:0005576)	47	5.95	7.90	1.26E-26
extracellular matrix (GO:0031012)	7	1.32	5.32	3.33E-03
PANTHER GO-Slim: Molecular Function				
serine-type endopeptidase inhibitor activity (GO:0004867)	8	.51	15.77	4.46E-06
peptidase inhibitor activity (GO:0030414)	10	.70	14.28	4.29E-07
serine-type peptidase activity (GO:0008236)	11	.81	13.60	1.74E-07
antigen binding (GO:0003823)	9	.75	12.02	4.73E-06
lipid transporter activity (GO:0005319)	4	.37	10.69	1.43E-02
peptidase activity (GO:0008233)	25	3.31	7.56	2.17E-12
receptor binding (GO:0005102)	27	7.51	3.59	4.72E-07
hydrolase activity (GO:0016787)	31	15.55	1.99	4.39E-03

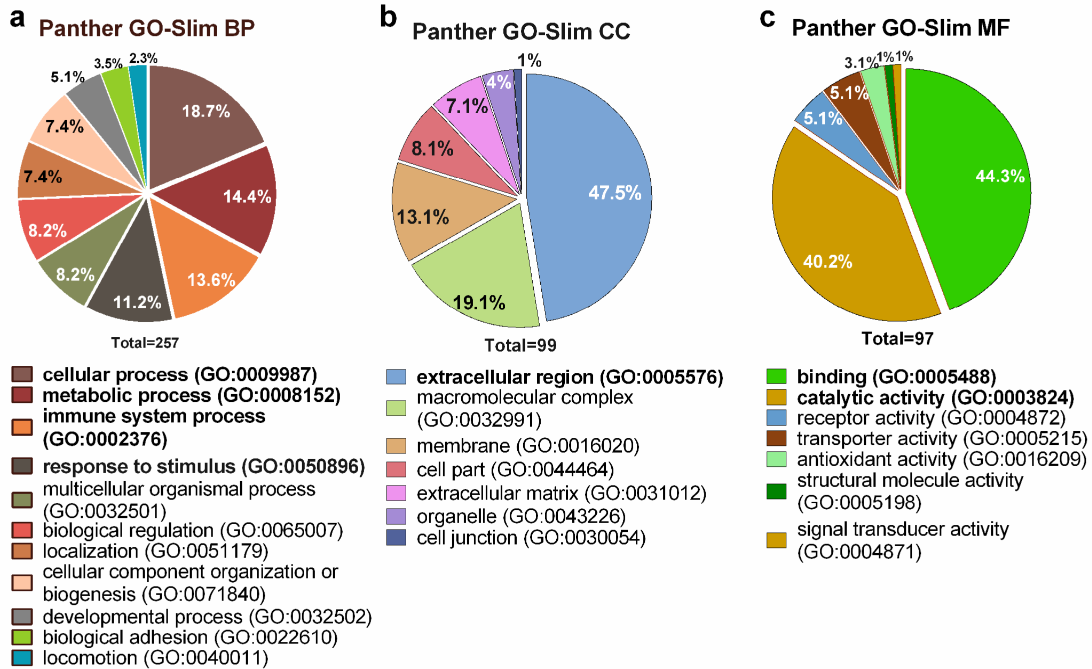
557

558 FIGURES



559
560 **Figure 1. Experimental workflow.** Samples from the different groups (S1-S6), and the
561 pooled sample (PS, S7) were firstly delipidated. Then, low abundance proteins were
562 enriched by means of ProteoMiner. The eluted fractions (EFs S1-S7) were then cleaned
563 and quantified, and 50 µg of protein were reduced, alkylated and trypsinized. Tryptic
564 peptides were labelled with different isobaric tags (113-118, 119, and 121) and
565 afterwards, all samples were pooled in one mixed sample (MS). The MS was analysed
566 three times by LC-MS/MS. Finally, all reporter intensities were normalized by the PS.

567



568

569 **Figure 2. GO annotations of all proteins identified in this study.** Two hundred and
570 seventeen proteins were identified in this study and functionally classified by biological
571 process (GO-Slim BP), cellular component (GO-Slim CC), and molecular function (GO-
572 Slim MF). The most abundant GO terms are highlighted in bold. Percentages are
573 displayed in graphs.

574

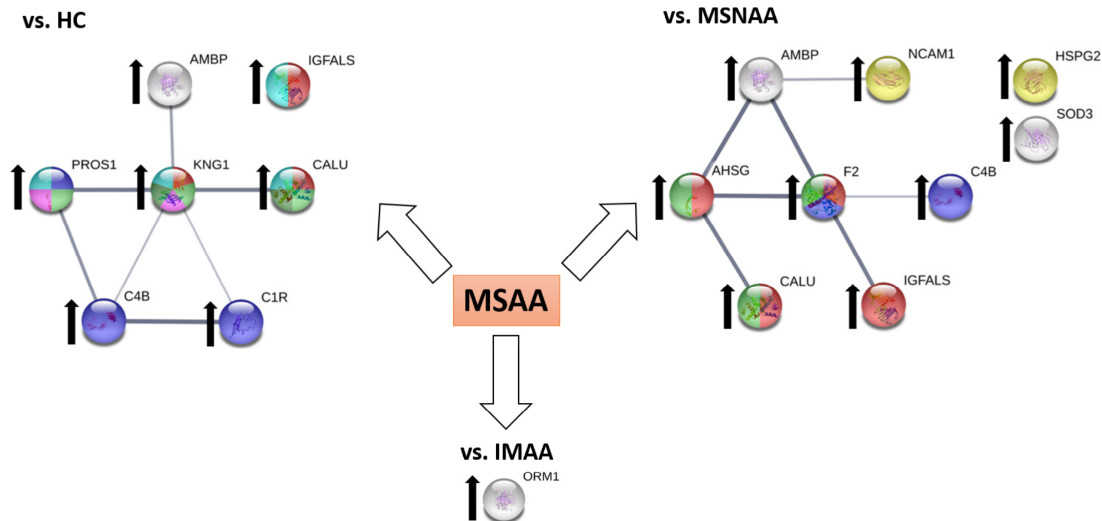


Figure 3. String analysis of differentially abundant proteins in MSAA patients compared to HC, MSNAA, and IMAA donors. Analysis of the protein interaction network was done by means of the STRING database. The thickness of the lines indicates the confidence level of the predicted interactions (high confident score / >0.9, thick lines; medium confident score / >0.4, thin lines). Colour legend represents the REACTOME pathways with overrepresentation against human proteome with an FDR < 0.01. The following abbreviations were used: moderate-severe allergic asthmatics, MSAA; moderate-severe non-allergic asthmatics, MSNAA; intermittent-mild allergic asthmatics, IMAA; healthy controls, HC; Alpha-1-Microglobulin/Bikunin Precursor, AMBP; Insulin-like growth factor-binding protein complex acid labile subunit, IGFALS; Protein S isoform 2 (Fragment), PROS1; Kininogen-1, isoform CRA_a, KNG1; Isoform 3 of Calumenin (Crocalbin), CALU; Complement C4B1a (Fragment), C4B; Complement C1r subcomponent, C1R; Alpha-1-acid glycoprotein 1 (AGP 1) (Orosomucoid-1) (OMD 1), ORM1; Neural cell adhesion molecule 1, NCAM1; AHSG; Prothrombin (Coagulation factor II), F2; Basement membrane-specific heparan sulfate proteoglycan core protein, HSPG2; Extracellular superoxide dismutase [Cu-Zn] (EC-SOD), SOD3.

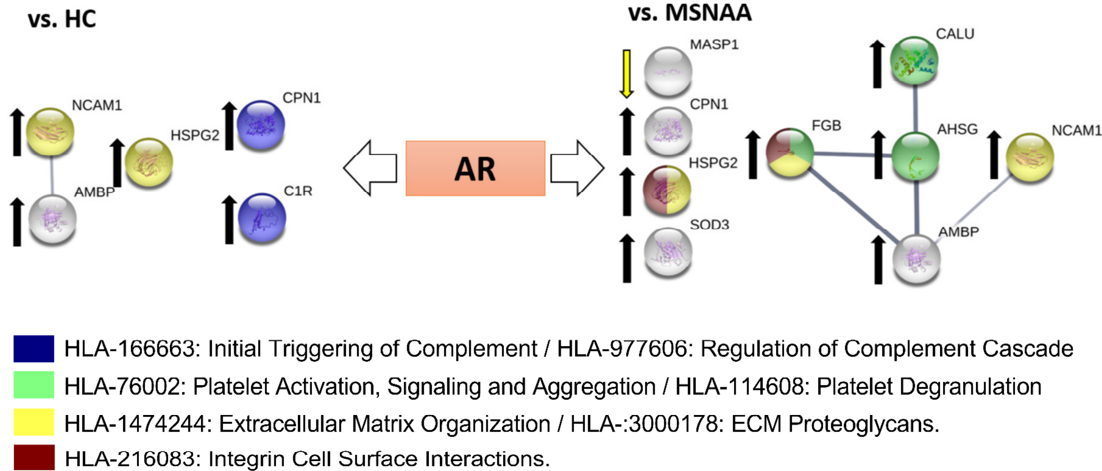
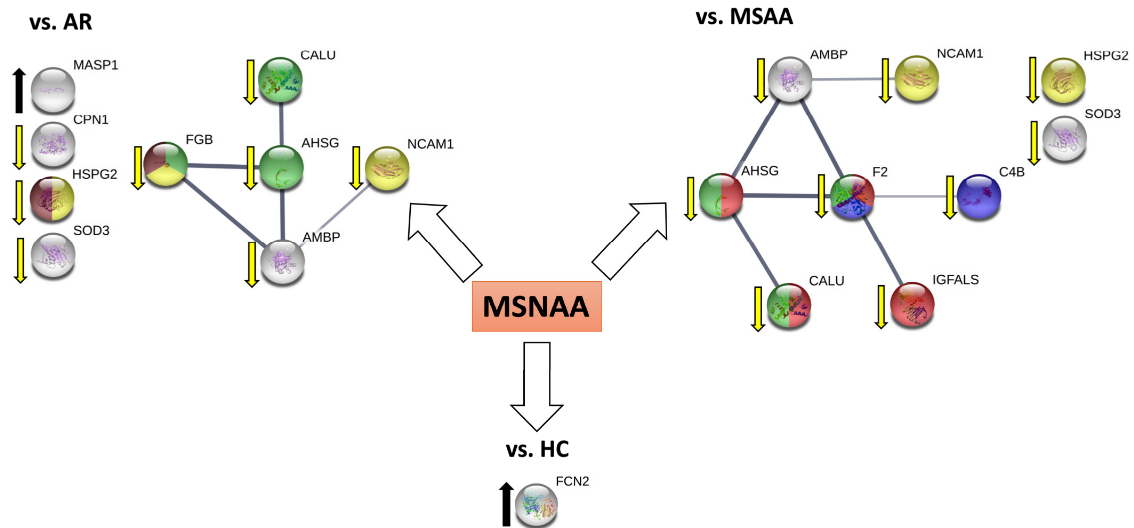


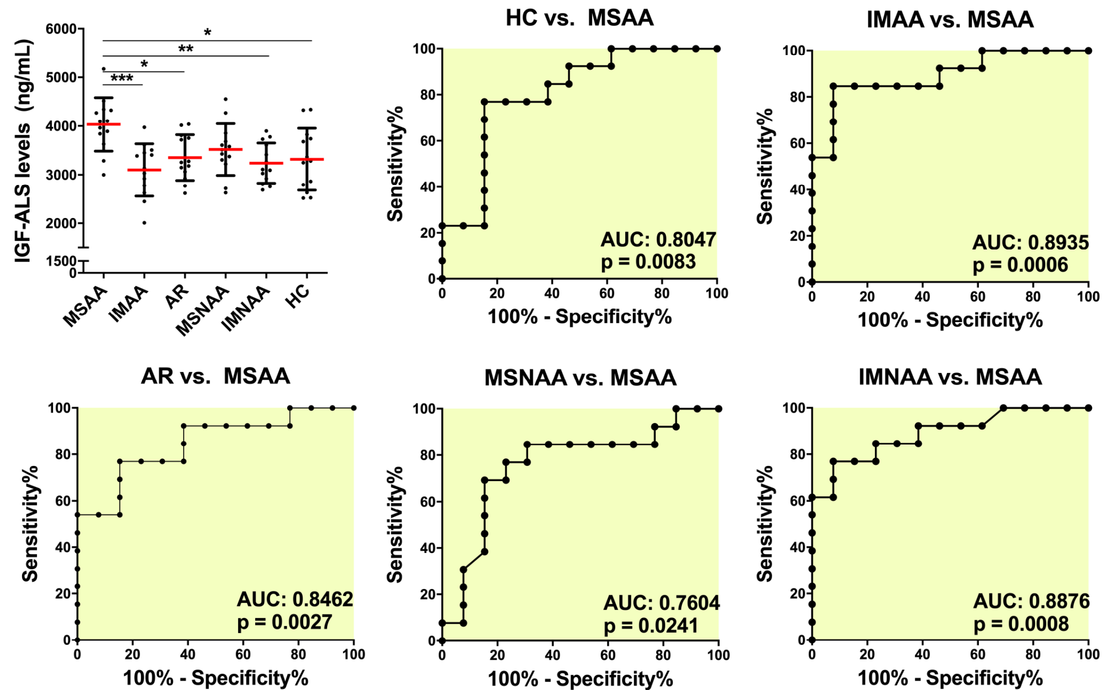
Figure 4. String analysis of differentially abundant proteins in allergic rhinitis patients compared to HC and MSNAA patients. Analysis of the protein interaction network was done by means of the STRING database. The thickness of the lines indicates the confidence level of the predicted interactions (high confident score / >0.9, thick lines; medium confident score / >0.4, thin lines). Colour legend represents the REACTOME pathways with overrepresentation against human proteome with an FDR < 0.01. The following abbreviations were used: allergic rhinitis patients, AR; moderate-severe non-allergic asthmatics, MSNAA; healthy controls, HC; NCAM; Alpha-1-Microglobulin/Bikunin Precursor, AMBP; Basement membrane-specific heparan sulfate proteoglycan core protein, HSPG2; Carboxypeptidase N catalytic chain (CPN), CPN1; Complement C1r subcomponent, C1R; Mannan-binding lectin serine protease 1, MASP1; Extracellular superoxide dismutase [Cu-Zn] (EC-SOD), SOD3; Fibrinogen beta chain, FGB; Isoform 3 of Calumenin (Crocalbin), CALU; cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein (Fetuin-A), AHSG; Alpha-1-Microglobulin/Bikunin Precursor, AMBP; Neural cell adhesion molecule 1, NCAM1.



HLA-381426: Regulation of Insulin-Like Growth Factor (IGF) Transport and Uptake by IGF-Binding Proteins.
HLA-166663: Initial Triggering of Complement / HLA-977606: Regulation of Complement Cascade
HLA-76002: Platelet Activation, Signaling and Aggregation / HLA-114608: Platelet Degranulation
HLA-1474244: Extracellular Matrix Organization / HLA-:3000178: ECM Proteoglycans.
610 HLA-216083: Integrin Cell Surface Interactions.

611 **Figure 5. String analysis of differentially abundant proteins in MSNAA patients**
612 **compared to HC and MSAA patients.** Analysis of the protein interaction network was
613 done by means of the STRING database. The thickness of the lines indicates the
614 confidence level of the predicted interactions (high confident score / >0.9, thick lines;
615 medium confident score / >0.4, thin lines). Colour legend represents the REACTOME
616 pathways with overrepresentation against human proteome with an FDR < 0.01. The
617 following abbreviations were used: moderate-severe allergic asthmatics, MSAA;
618 moderate-severe non-allergic asthmatics, MSNAA; healthy controls, HC; Mannan-
619 binding lectin serine protease 1, MASP1; Carboxypeptidase N catalytic chain (CPN),
620 CPN1; Basement membrane-specific heparan sulfate proteoglycan core protein HSPG2;
621 Extracellular superoxide dismutase [Cu-Zn] (EC-SOD), SOD3; Fibrinogen beta chain,
622 FGB; Isoform 3 of Calumenin (Crocalbin), CALU; cDNA FLJ55606, highly similar to
623 Alpha-2-HS-glycoprotein (Fetuin-A), AHSG; Alpha-1-Microglobulin/Bikunin Precursor,
624 AMBP; Neural cell adhesion molecule 1, NCAM1; Ficolin-2 (Hucolin), FCN2;
625 Prothrombin (Coagulation factor II), F2; Complement C4B1a (Fragment), C4B; Insulin-
626 like growth factor-binding protein complex acid labile subunit, IGFALS.

627



628

629 **Figure 6. Validation of iTRAQ results by ELISA and ROC analysis.** a) Thirteen serum
630 samples from each donors group (moderate-severe allergic asthmatics, MSAA;
631 intermittent-mild allergic asthmatics, IMAA; allergic rhinitis patients, AR; moderate-
632 severe non-allergic asthmatics, MSNAA; intermittent-mild non-allergic asthmatics,
633 IMNAA; and healthy controls, HC) were selected to achieve a similar average age and
634 M/F proportion in all of them. Then, IGFALS protein was measured by ELISA. The
635 asterisk (*) indicates the presence of a significant difference. * $P < 0.05$ ** $P < 0.01$ *** P
636 < 0.001. b-f) The diagnostic ability of IGFALS to discriminate the different classes of
637 donors was also evaluated through different receiver/relative operating characteristic
638 (ROC) curves. An area under the ROC curve (AUC) > 0.8 was considered as a good
639 performance of the biomarker.