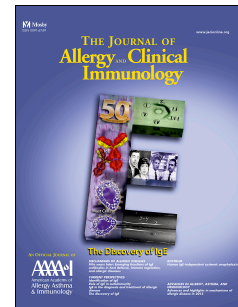


Accepted Manuscript

Sialylated Fetuin-A as a candidate predictive biomarker for successful grass pollen allergen immunotherapy

Noémie Caillot, PhD, Julien Bouley, PhD, Karine Jain, MSc, Sandrine Mariano, PhD, Sonia Luce, MSc, Stéphane Horiot, MSc, Sabi Airouche, MSc, Chloé Beuraud, PhD, Christian Beauvallet, PhD, Philippe Devillier, MD, Sylvie Chollet-Martin, PhD, Christine Kellenberger, PhD, Laurent Mascarell, PhD, Henri Chabre, PhD, Thierry Batard, PhD, Emmanuel Nony, PhD, Vincent Lombardi, PhD, Véronique Baron-Bodo, PhD, Philippe Moingeon, PhD



PII: S0091-6749(16)31450-6

DOI: [10.1016/j.jaci.2016.10.036](https://doi.org/10.1016/j.jaci.2016.10.036)

Reference: YMAI 12500

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

Received Date: 1 March 2016

Revised Date: 19 August 2016

Accepted Date: 12 October 2016

Please cite this article as: Caillot N, Bouley J, Jain K, Mariano S, Luce S, Horiot S, Airouche S, Beuraud C, Beauvallet C, Devillier P, Chollet-Martin S, Kellenberger C, Mascarell L, Chabre H, Batard T, Nony E, Lombardi V, Baron-Bodo V, Moingeon P, Sialylated Fetuin-A as a candidate predictive biomarker for successful grass pollen allergen immunotherapy, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2016.10.036.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Sialylated Fetuin-A as a candidate predictive biomarker for successful grass pollen allergen immunotherapy

Noémie Caillot^{1*}, PhD, Julien Bouley^{1*}, PhD, Karine Jain¹, MSc, Sandrine Mariano¹, PhD, Sonia Luce¹, MSc, Stéphane Horiot¹, MSc, Sabi Airouche¹, MSc, Chloé Beuraud¹, PhD, Christian Beauvallet², PhD, Philippe Devillier³, MD, Sylvie Chollet-Martin⁴, PhD, Christine Kellenberger⁵, PhD, Laurent Mascarell¹, PhD, Henri Chabre¹, PhD, Thierry Batard¹, PhD, Emmanuel Nony¹, PhD, Vincent Lombardi¹, PhD, Véronique Baron-Bodo¹, PhD, and Philippe Moingeon¹, PhD.

¹ Research Department, Stallergenes Greer, Antony, France

² INRA, UMR 1313 GABI, Research center, Jouy-en-Josas, France

³ UPRES EA 220 and Clinical Research Department, Foch Hospital, Suresnes, France

⁴ INSERM, UMR 996, Paris-Sud University, Châtenay-Malabry, France

⁵ CNRS, UMR 6098, AFMB, Marseille, France

* These authors contributed equally to this work

Correspondence: Philippe Moingeon, Research Department, Stallergenes Greer, 6 rue Alexis de Tocqueville, 92160 Antony, France, Tel: +33 1 55 59 25 20, e-mail: pmoingeon@stallergenes.com

Conflict of interest statement: Several co-authors (NC, JB, KJ, SM, JC, SL, SH, HC, TB, EN, VL, VB-B, PM) are employees at Stallergenes Greer.

Abbreviations used:

- AHSG: Alpha-2-HS-glycoprotein
- AIT: Allergen immunotherapy
- ANR: Active non-responder
- AR: Active responder
- ARTSS: Average rhinoconjunctivitis total symptom score
- BMK: Biomarker
- DC: Dendritic cell
- DC1: DC driving the differentiation of T_H1 cells
- DC2: DC driving the differentiation of T_H2 cells
- DCReg: DC driving the differentiation of regulatory T cells
- D. far*: *Dermatophagoides farinae*
- FetA: Fetuin A
- HDM: House dust mite
- LPS: Lipopolysaccharide
- MoDC: Monocyte-derived dendritic cell
- MS: Mass spectrometry
- mOG1: murine O-glycopeptide 1
- mOG2: murine O-glycopeptide 2
- mOG2-A or B: murine O-glycopeptide 2 A or B
- mOG3: murine O-glycopeptide 3
- OG1: O-glycopeptide 1
- OG2 : O-glycopeptide 2
- OG3: O-glycopeptide 3
- OVA: Ovalbumin

- 49 PNR: Placebo non-responder
- 50 PR: Placebo responder
- 51 ROC: Receiver operating characteristic
- 52 SLIT: Sublingual immunotherapy
- 53 TLR4: Toll-like receptor 4
- 54 2D-DiGE: Two-dimensional differential gel electrophoresis
- 55

Abstract

Background: Eligibility to immunotherapy is based on the determination of IgE reactivity to a specific allergen by skin prick or *in vitro* testing. Biomarkers predicting the likelihood of clinical improvement during immunotherapy would significantly improve patient selection.

Methods: Proteins were differentially assessed by 2D-DiGE and label-free mass spectrometry in pre-treatment sera obtained from clinical responders and non-responders within a cohort of 82 grass pollen allergic patients receiving sublingual immunotherapy or placebo. Functional studies of Fetuin-A were conducted by using both gene silencing in a mouse asthma model, human dendritic cell *in vitro* stimulation assays and surface plasmon resonance.

Results: Analysis by quantitative proteomics of pre-treatment sera from grass-pollen allergic patients reveals that high levels of O-glycosylated sialylated Fetuin-A isoforms are found in those patients exhibiting a strong decrease in rhinoconjunctivitis symptoms following sublingual immunotherapy. Whereas Fetuin-A is involved in numerous inflammatory conditions, its potential role in allergy is unknown. *In vivo* silencing of the *FETUA* gene in BALB/c mice results in a dramatic upregulation of both airway hyperresponsiveness, lung resistance, as well as T_H2 responses following allergic sensitization to ovalbumin. Both sialylated and non sialylated Fetuin-A bind to LPS, but only the former synergizes with LPS and grass pollen or mite allergens to enhance TLR4-mediated proallergic properties of human dendritic cells.

Conclusions: As a reflection of the patient's inflammation status, pre-treatment levels of sialylated Fetuin-A in the blood are indicative of the likelihood of clinical responses during grass pollen immunotherapy.

Clinical implications: FetA plays either an inhibiting or amplifying role in allergic inflammation, depending upon post-translational modifications of the molecule. Patients with

80 high levels of sialylated FetA glycoforms in their blood before treatment are more likely to
81 benefit from grass pollen immunotherapy.

ACCEPTED MANUSCRIPT

82

Capsule summary

83 Using non-hypothesis driven comparative proteomics, we identified sialylated Fetuin-A as a
84 novel candidate BMK of efficacy overexpressed in the blood of patients more likely to
85 respond to grass pollen immunotherapy.

86

87

88

Introduction

89 Allergen immunotherapy (AIT) performed *via* the subcutaneous or sublingual routes is
90 currently established as a safe and efficacious treatment for type I (IgE-mediated) respiratory
91 allergies to pollen and house dust mite allergens.¹⁻³ Potential biomarkers (BMKs) of efficacy
92 for AIT are generating considerable interest, since they could be used to inform the
93 physician's decisions during treatment.⁴⁻⁸ BMKs are commonly defined as indicators of either
94 normal or pathogenic processes, or of responses to a therapeutic intervention, which can be
95 objectively measured in readily accessible biological samples.⁴

96 The search for biomarkers of efficacy has been so far based on the paradigm that AIT acts by
97 shifting allergen-specific CD4⁺ T-cell responses from a T_H2 to a T_H1/regulatory T-cell
98 pattern.^{7, 9, 10} Thus, clinically efficacious AIT has been related to the induction of allergen-
99 specific regulatory T cells, associated with IgG4 responses encompassing high affinity
100 blocking antibodies competing with IgE for allergen binding.¹¹⁻¹³ Recently, we also reported
101 that the early onset of clinical efficacy is correlated with the induction in peripheral blood of
102 monocyte-derived regulatory dendritic cells (DCRegs) concomitantly with a decrease in
103 proallergic type 2 DCs (i.e., DC2s).^{14, 15} Although all those biological changes represent
104 candidate follow-up markers reflecting efficacy after AIT has been initiated, patient selection
105 would further benefit from the identification of BMKs predicting the likelihood of clinical
106 responsiveness before starting treatment.⁸

107 Patients' eligibility to immunotherapy is currently based on the determination of IgE
108 reactivity to a specific allergen by skin prick or *in vitro* testing. In addition, patients with
109 either severe rhinoconjunctivitis symptoms,^{16, 17} high basophil responsiveness to allergens,¹⁸
110 or elevated allergen-specific IgE titers¹⁹⁻²² prior to treatment appear more likely to benefit
111 from AIT. As of today, however, no marker of AIT efficacy has been firmly established,

which could be used at an individual patient level to predict positive immunotherapy outcomes.

Herein, we searched for such BMKs of sublingual immunotherapy (SLIT) efficacy in sera from 82 grass pollen allergic patients enrolled in a double-blind placebo-controlled study performed in an exposure challenge chamber.²³ Using comparative proteomics, we provide evidence for an increased O-linked sialylation of Fetuin-A (FetA) in sera collected before treatment from patients exhibiting clinical responses, when compared with low responders. We also demonstrate that FetA plays a role in the regulation of allergic inflammation and further, that sialylated FetA, but not its desialylated counterpart (termed AsialoFetA), enhances TLR4-mediated allergic inflammation.

Methods

Clinical samples from the VO56.07A pollen chamber study

Details of the VO56.07A clinical study have been published elsewhere.²³ Briefly, 82 grass pollen allergic patients were randomized to receive once daily for 4 months either a grass pollen (n = 42) or placebo (n = 40) tablet through the sublingual route. Patients were exposed to grass pollen grains in a chamber (Allergy center in Vienna West, Austria) before treatment, as well as after 1 week and 1, 2, and 4 months of treatment.²³ Clinical responses were evaluated following allergen challenge for each individual patient as percentages of improvement in Average Rhinoconjunctivitis Total Symptom Score (ARTSS) measured as described elsewhere.²³ As a first step, the median percentage of ARTSS improvement in the active group after treatment was used as an arbitrary threshold to identify clinical responders, with patients exhibiting a $\geq 43.9\%$ ARTSS improvement considered as responders, and those with a lower improvement as non-responders. Based on this threshold, 4 patient subgroups were defined, including active responders (AR, n = 21), active non-responders (ANR, n = 21), placebo responders (i.e., patients exhibiting clinical improvement unrelated to treatment efficacy, PR, n = 7), and placebo non-responders (PNR, n = 33). Sera were collected from patients before and after AIT, and kept frozen at -80°C until use. All biological analyses were performed on samples coded to the operators.

Identification and characterization of FetA and sialylated FetA

Proteomes were assessed using quantitative proteomics in serum samples from clinical responders and non-responders. Serum sample depletion, 2-Dimensional Differential Gel Electrophoresis (2D-DiGE) and mass spectrometry (MS) analysis of differentially expressed

spots, as well as label-free MS-based proteomics are described in details in the Methods section of this article's Online Repository at www.jacionline.org.

Purification and MS analysis of human and murine FetA, as well as the FetA desialylation procedure are described in the Methods section of the Online Repository. Functional properties of FetA were analyzed in OVA or grass pollen-sensitized BALB/c mice after *in vivo* gene silencing prior to measurement of airway hyper-responsiveness and lung resistance. FetA and AsialoFetA were compared for their capacity to activate human monocyte-derived dendritic cells (DCs), alone or in combination with LPS and allergen extracts. LPS-FetA-TLR4 interactions were measured by surface plasmon resonance. All *in vivo* and *in vitro* functional assays are described in the Methods section of this article's Online Repository at www.jacionline.org.

Statistics

Data are expressed as means $\pm$ SEMs. Differences in protein expression or peptide abundance, observed by 2D-DiGE analysis or label-free MS experiments, respectively, were analyzed for statistical significance applying an ANOVA p-value threshold of 0.01, with a fold-change filter ≥ 1.5 . Statistics on proteomic data were performed with the Samespots or Progenesis LC-MS software from Nonlinear Dynamics (Newcastle upon Tyne, UK). Correlation analyses were performed by using the non-parametric Spearman test. P-values < 0.05 were considered as significant. Statistical differences between groups were assessed by using either 2-tailed nonparametric Mann-Whitney tests, 2-tailed parametric t-tests and the Friedman test for multiple comparisons. The Kruskal-Wallis and the two-way ANOVA tests were used in murine experiments for the analyses of cytokine production and airway hyper-reactivity measurements, respectively. Statistical analyses and graphical representations were performed with the GraphPrism 6 software (GraphPad Software, Inc, La Jolla, USA).

Results

Identification by quantitative proteomics of BMKs predictive of SLIT efficacy

To identify markers predicting the clinical response to AIT, we combined 2D-DiGE and MS in order to assess differential protein expression in pre-treatment sera obtained from 82 grass pollen allergic patients receiving either a grass pollen (n = 42) or placebo (n = 40) tablets sublingually, once a day, for 4 months (Supplementary Table E1, Supplementary Figure E1). As a first step, we applied an arbitrary threshold corresponding to the median percentage of ARTSS improvement in the active group after treatment in order to stratify patients in 4 subgroups (cf. Methods) including active responders (ARs, n = 21), active non-responders (ANRs, n = 21), placebo responders (PRs, n = 7), and placebo non-responders (PNRs, n = 33). Serum samples from these subgroups were compared in a blind manner by using 2D-DiGE (**Figure 1A**), after immunoaffinity depletion of the 6 most represented proteins to improve the quantification of less abundant proteins. Differentially expressed protein spots were excised from 2D-gels, digested with trypsin and analyzed by MS. In such analyses, differences in levels of expression of the FetA protein (also termed Alpha-2-HS-glycoprotein, AHSG) were observed between pre-treatment sera obtained from AR and ANR patients (**Figure 1A, B** and Supplementary Table E2). Specifically, four FetA proteoforms (corresponding to spots sp419, 428, 439 and 448) were highly expressed in pre-treatment sera of AR relative to ANR individuals ($p < 0.01$ to 0.001 , **Figure 1B, and 1C left panel**), whereas the proteoform present in spot 469 was rather decreased ($p < 0.05$, **Figure 1B and 1C, left panel**). No such differences were observed in the placebo group (**Figure 1D, left panel**).

To validate those results obtained after patient stratification based on an arbitrary threshold of efficacy, percentages of symptom score improvement for each individual patient were then plotted against the abundance of the sum of acidic spots 428, 439 and 448. These parameters

were highly significantly correlated (with a Spearman correlation $r = 0.50$, $p = 0.0009$) within the cohort of patients receiving the active treatment (**Figure 1C, right panel**), whereas no such a correlation was observed in placebo-treated patients (**Figure 1D, right panel**). Noteworthy, we did not observe any variation in serum levels of FetA acidic forms in the course of AIT (**Figure 1E**), establishing that patterns of FetA proteoforms are tightly regulated as a characteristic of each individual patient. Collectively, these results suggest differences in the relative distribution of FetA proteoforms in pre-treatment sera of AR patients when compared with ANRs.

O-glycosylated sialylated FetA is highly abundant in pre-treatment sera of clinical responders

FetA is a 60 kDa heterodimer between a 282 amino acid A chain and a 27 amino acid B chain assembled by a single disulfide bond. The protein bears both N- and O-linked oligosaccharide chains containing sialic acid residues (UniProtKB accession number P02765).^{24, 25} To further characterize human FetA proteoforms, we purified the molecule from sera of healthy individuals by affinity chromatography (Supplementary Figure E2) and conducted MS analyses. The later confirmed the presence of multiple FetA proteoforms bearing sialic acids (i.e., N-acetylneuraminic acid, noted Neu5Ac) branched on N- and O-glycan moieties, both on the A and B chains (**Figure 2A** and Supplementary Figure E3). The treatment of purified FetA with α -(2→3,6,8,9)-neuraminidase to remove sialyl residues (Supplementary Figure E4) resulted in a complete shift of the spot charge-train in the 2D-gel profile (**Figure 2B**, comparing FetA in green with AsialoFetA in red), thereby confirming that the presence of Neu5Ac results into a more acidic pI for the molecule. Although not shown, the heterogeneity of desialylated FetA is also due to various levels of serine and threonine phosphorylation.

In order to further characterize its glycosylation pattern, purified FetA was analyzed by LC-MS/MS following digestion by trypsin. Tryptic glycopeptides were assigned based on

accurate precursor and specific fragment ion mass measurements. For example, glycopeptides containing sialylated glycans yield b-type ions with neutral masses of 291.1 and 273.1 Da (corresponding to Neu5Ac and dehydrated Neu5Ac, respectively). Among the 5 glycosylation sites described in the literature,²⁴ residues N₁₅₆ and N₁₇₆ were confirmed to be substituted with N-glycan moieties harboring two sialic acid residues (i.e., Hex₅HexNAc₄Neu5Ac₂, Supplementary Table E3). Interestingly, O-glycopeptides from the A chain bear two mucin-type glycans branched on residues T₂₅₆ and T₂₇₀, each sialylated with one or two Neu5Acs, yielding variants with 2, 3 or 4 Neu5Acs subsequently termed O-glycopeptide 1 (OG1), O-glycopeptide 2 (OG2), and O-glycopeptide 3 (OG3), respectively (**Figure 2C** and Supplementary Table E3). In addition, the B chain also encompasses both non-, mono- and disialylated forms with Neu5Ac attached to residue S₃₄₆ (Supplementary Figure E3, B-D).

To confirm whether the shift toward acidic forms of FetA observed in AR patients is related to sialylation levels, a MS analysis of FetA glycopeptides was conducted in sera from allergic patients (Supplementary Table E3). O-glycopeptides containing two Neu5Acs were significantly increased in pre-treatment sera from AR relative to ANR patients (including OG2; $p < 0.05$; OG3, $p < 0.001$, **Figure 3A, left panel**; disialylated B chain, $p < 0.01$, Supplementary Table E3). Importantly, abundances of the tetrasialylated glycopeptide OG3 (and to a lower extent of trisialylated glycopeptide OG2) before treatment were significantly correlated with clinical benefit in patients from the active group (with a Spearman correlation $r = 0.55$, $p = 0.0002$, **Figure 3A, right panel**), whereas no such correlations were observed in placebo-treated patients (**Figure 3B**). As shown in Supplementary Figure E5 in the Online Repository, a highly significant correlation was observed between OG3 peptide abundance and spot volumes (sp 428 + 439 + 449) in pre-treatment sera of individual patients. Although not shown, total serum FetA concentrations (assessed by either ELISA or label-free MS) were not correlated with clinical benefit, confirming that the predictive value is strictly linked to

levels of O-glycosylated sialylated FetA. As shown in **Figure 3C**, a receiver operating curve (ROC) of OG3 abundance confirmed that this marker is useful to distinguish clinical responders from non-responders (with an area under the curve AUC of 0.8050 and a p-value of 0.0007).

In vivo silencing of the FETUA gene in BALB/c mice exacerbates allergen-induced inflammation

FetA is known as an acute-phase protein with either a protective or enhancing role in various forms of inflammation.²⁵ As of today, however, no specific role in allergic inflammation has been assigned to FetA. To investigate the potential effect of FetA in a murine model of allergic asthma, the *FETUA* gene was inactivated in BALB/c mice sensitized with ovalbumin (OVA). To this aim, a group of animals received multiple intravenous injections of a FetA-specific vivo-morpholino (**Figure 4A**), using naïve (i.e., non-sensitized) or untreated sensitized mice as well as animals receiving an irrelevant morpholino as controls in those experiments. The efficacy of FetA silencing was confirmed by ELISA, with a 55.7 +/- 13.3 % (mean +/- SEM) decrease in FetA serum concentrations observed at day 9 compared to pre-treatment values ($p < 0.05$) in animals receiving the FetA-specific vivo-morpholino (**Figure 4B**). In contrast, levels of FetA remained stable over time in sera from naïve or mock-treated animals (**Figure 4B**). FetA silencing resulted in exacerbated airway hyperresponsiveness (measured as a Penh index) when compared with either naïve mice, untreated sensitized mice or OVA-sensitized animals receiving the control morpholino ($p < 0.0001$, **Figure 4C**). As shown in Supplementary Figure E6 in the Online Repository, similar results were obtained in grass pollen-sensitized mice. These results were further confirmed by invasive plethysmography, since animals with the *FETUA* gene inactivated exhibited a statistically significant ($p < 0.05$) increase in lung resistance compared to control animals (**Figure 4D**). In

agreement with those results, the production of IL-5, IL-10 and IL-13 T_H2 cytokines was significantly ($p < 0.01$) increased in splenocytes from FetA-silenced mice restimulated *in vitro* with OVA, when compared with sensitized animals receiving the control morpholino (**Figure 4E**).

Sequence alignment revealed that murine FetA shares 59% amino acid identity with its human counterpart (Supplementary Figure E7). Whereas human FetA bears 3 O-glycosylation sites, murine FetA displays a single site (T_{267}), either unsialylated or bearing one or two N-glycolylneuraminic acids (Neu5Gc, as opposed to Neu5Ac found in human FetA), thus yielding variants with either 0, 1 or 2 Neu5Gcs termed murine O-glycopeptide 1 (mOG1), O-glycopeptides 2 A or B (mOG2-A or -B), and O-glycopeptide 3 (mOG3), respectively (**Figure 4F**, to be compared to **Figure 2C**).

FetA, but not AsialoFetA, synergizes with LPS in a TLR4-dependent pathway to enhance the proallergic profile of human monocyte-derived dendritic cells (MoDCs)

FetA has been shown to promote lipid-mediated inflammation by acting as an adaptor molecule between free fatty acids and toll-like receptor 4 (TLR4).²⁶⁻²⁸ The engagement of TLR4 is known to enhance allergic inflammation, for example during concomitant exposure to allergens in presence of endotoxins,²⁹⁻³¹ or as a consequence of a functional mimicry of MD-2 by the allergen itself.³² In this context, we examined whether FetA could contribute to allergic inflammation in a TLR4-dependent manner, and further whether FetA sialylation would play a potential role in this regard. In a first set of experiments, functional interactions between FetA and TLR4 were examined using HEK-293 cells expressing both human TLR4 as well as a reporter alkaline phosphatase gene inducible following TLR activation. Those hTLR4 HEK-293 cells were first stimulated with either LPS, FetA at various doses, or a mixture of both. As shown in **Figure 5A**, FetA alone had no effect, whereas a combination of

FetA and LPS acted in synergy to engage TLR4 in a dose-dependent manner. We subsequently compared FetA and AsialoFetA (i.e., neuraminidase-treated FetA, with complete desialylation confirmed by LC-MS; Supplementary Figure E4) for their capacity to synergize with LPS in this cellular assay. The synergistic modulation of TLR4 activity by LPS was substantially decreased when hTLR4 HEK-293 cells were costimulated with AsialoFetA, as opposed to FetA (**Figure 5B**).

To investigate further the potential impact of FetA sialyloforms on TLR4-mediated allergic inflammation, immature MoDCs were cultured in serum free medium (to preclude exposure to FetA from bovine serum) supplemented with a mixture of histamine, IL-25, IL-33, prostaglandin E₂, TSLP, and low doses (10 ng/mL) of LPS. In our hands, those conditions (termed the “DC2 cocktail”) drive the differentiation of MoDCs towards a DC2 phenotype (i.e., capable to polarize naïve CD4⁺ T cells towards IL-5 and IL-13 secreting T_H2 cells).¹⁵ A flow cytometry analysis confirmed an up-regulation in such DCs of CD83 and CD86 co-stimulatory markers in presence of FetA, but not AsialoFetA (**Figure 5C**). Similarly, DCs differentiated in presence of FetA secreted significantly higher levels of IL-6, IL-8, IL-10 and TNF- α , when compared to AsialoFetA-treated DCs (**Figure 5D**). Noteworthy, the synergy observed between LPS and FetA in those assays was totally abrogated by adding the TLR4 antagonist LPS-RS³³ (data not shown), confirming that FetA and LPS act in combination to activate the TLR4 pathway. Furthermore, the expression by DCs of a gene associated with DC2 polarization (i.e., PADI2)¹⁵ was upregulated in presence of FetA, but not AsialoFetA (**Figure 5E**). In contrast, the expression of genes rather associated with DC1 or DCReg differentiation, such as *MX1* or *C1QA*, respectively,¹⁴ was strongly down-regulated when DCs were cultured in presence of FetA, but not AsialoFetA (**Figure 5E**). In addition, following stimulation of immature MoDCs with a grass pollen extract or a house dust mite (HDM) *Dermatophagoides farinae* (*D. far*) extract, in combination with either FetA or

AsialoFetA, only the former synergized with the allergen extracts to induce the secretion of IL-6, IL-10 and TNF- α cytokines, whereas no such effect was observed with AsialoFetA (**Figure 5F, G**). A synergy with FetA, but not AsialoFetA, was also detected for IL-8 secretion by immature MoDCs, when stimulating with the grass pollen, in contrast to the *D. far* allergen extract, since levels of IL-8 produced were already very high with the latter allergen alone (**Figure 5F, G**). Altogether, those experiments establish that FetA can enhance the proallergic profile of DCs exposed to natural allergens, in relationship with its level of sialylation.

We subsequently investigated by surface plasmon resonance the potential physical interactions between FetA (or AsialoFetA), LPS and TLR4-MD2. Both FetA and AsialoFetA were found to bind LPS in a comparable dose-dependent manner (**Figure 6A, B**). In contrast, neither FetA nor AsialoFetA had any detectable impact on the physical interaction of LPS with TLR4-MD2 complexes (**Figure 6C-E**).

Discussion

AIT is a well-established treatment for type I respiratory allergies, inducing a long-lasting reduction of symptoms in association with a down-regulation of allergen-specific T_H2 responses.^{1-3, 9-11, 34} As of today, however, less than one third of patients who could benefit from AIT are proposed this therapy,³⁵ on the basis of their symptom severity, reactivity to skin prick tests and/or high titers of allergen-specific IgEs.^{1, 16-22} Patient selection and compliance would significantly be improved if predictive biological markers were available to identify those individuals more likely to benefit from AIT.

To address this need, we performed a comparative proteomic analysis on pre-treatment sera obtained from 82 grass-pollen allergic patients included in a double-blind placebo-controlled clinical study performed in an allergen challenge chamber.²³ In this study, up to 30% symptom score improvement was observed in the active group relative to placebo after 4 months of sublingual AIT.²³ Importantly, symptom scoring of each individual patient before and after AIT allowed to distinguish clinical responders from non-responders. Comparative analyses of serum proteomes by 2D-DiGE combined with LC-MS/MS established that FetA acid glycoforms are differentially expressed before treatment in sera from ARs relative to ANRs. Consistent with the known existence of post-translational modifications on FetA,^{24, 25, 36-38} we established that sialic acid (Neu5Ac) residues are responsible for the shift towards acidic pI of FetA isoforms observed in sera from AR patients. A comprehensive MS characterization further confirmed that levels of tetrasialylated O-glycopeptide 3 from FetA before treatment positively correlate ($p < 0.0002$) with clinical improvement during subsequent AIT.

FetA is an abundant blood protein produced in the liver, with multiple functions linked to its capacity to bind ligands as diverse as calcium, TGF β , the insulin receptor, free fatty acids and TLR4.²⁵ As a mineral carrier protein, it is involved in the regulation of osteogenesis and the

prevention of vascular calcification.^{39, 40} FetA also inhibits the insulin receptor tyrosine kinase activity, contributing to metabolic disorders such as insulin resistance and dyslipidemia.^{27, 41} In murine models, FetA often mediates a protective anti-inflammatory activity, for example against cerebral ischemic injury or lethal sepsis.^{42, 43} In human pathology, the protein is considered either as a negative or positive acute phase protein, as many inflammatory conditions are associated with either a decrease (e.g., trauma, rheumatoid arthritis, infection, kidney disease, hematological malignancies, myocardial infarction)^{25, 37, 44-46} or an increase (e.g., obesity, type 2 diabetes, ischemic stroke)^{25, 47-49} in serum FetA levels. Although measurement of FetA in the blood is already used as a prognostic marker for some of those conditions, little is known about the significance of its post-translational modifications. Also, whereas the existing body of scientific literature supports the notion that FetA may act either as an inhibitor or promoter of chronic inflammation, up to now no specific role had been ascribed to it in T_H2-mediated allergic inflammation.

Herein, we demonstrate that silencing of the gene encoding FetA in a murine model of allergic asthma results into an exacerbated allergic inflammation, thus revealing a strong anti-inflammatory activity of FetA in this experimental setting. This observation is however not easily transposable to human allergies, since murine and human FetA molecules differ significantly, with only 59% homology in their amino acid sequences (Supplementary Figure E5). Also, murine FetA lacks the sialylated O-glycans found in the human protein (cf. **Figure 2C** and **Figure 4F**). When testing the functionality of human FetA, we established in both hTLR4 HEK-293 and human DC models that FetA synergizes with LPS to activate TLR4, promoting as a consequence both the differentiation of MoDCs into proallergic DC2s, the secretion of pro-inflammatory cytokines as well as the down-regulation of genes associated with DC1 or DCRegs. Further supporting a role of human FetA in allergic inflammation, the latter was found to increase TLR4 activation by grass pollen or HDM extracts, known to

contain both allergens and endotoxins.^{29, 30, 50} Interestingly, this synergistic activation of the TLR4 pathway was largely diminished for neuraminidase-treated FetA, highlighting the key role of sialic acids branched on glycans in this proallergic cascade. In agreement with the recent identification of FetA as a central adaptor molecule contributing to fatty acid-mediated inflammation, our study confirms the capacity of FetA to interact with the TLR4 pathway and induce NF κ B-driven genes.²⁷ Surface plasmon resonance documented that both FetA and AsialoFetA bind similarly to LPS, without evidence in our hands for any strong binding to the TLR4-MD-2 complex. How sialylation of FetA influences the functional activation of TLR4 is presently unclear, but is consistent with a previous observation that removal of N- and O-linked β -galactosides bearing sialic acid residues abrogates the interaction of FetA with TLR4.²⁷ Noteworthy, a reduced sialylation of blood FetA has been reported in neonates with intrauterine growth restriction⁵¹ and in patients with rheumatoid arthritis.³⁸

Despite the new evidence presented in this study that sialylated human FetA amplifies allergic inflammation, it is still unclear why the persistence of sialylated forms of FetA in patients' sera might be predictive of clinical benefit during AIT. A high degree of FetA sialylation could reflect a higher reactivity to allergens due to a stronger engagement of the pro-inflammatory TLR4 pathway. Indeed, patients with high levels of sialylated FetA tend to exhibit more severe rhino-conjunctivitis symptoms before treatment (data not shown), which is known to favor clinical improvement during AIT.¹⁷ One potential limitation to the use of FetA as a biomarker predictive of AIT efficacy is that this protein plays a ubiquitous role in many aforementioned inflammatory conditions associated with quantitative variations of blood FetA levels.^{25, 44-49} Remarkably, however, we observed highly stable levels of FetA acidic variants in the blood of grass pollen patients over the 4 months of our study, irrespective of allergen exposure either in the pollen chamber or during the course of AIT. This suggests that levels of sialylated FetA in the blood are tightly regulated and may reflect

410 the own capacity of each individual patient to mount and/or resolve inflammatory reactions.
411 Validation of the hypothesis that a marker associated with “an inflammation status” can be
412 used to predict AIT efficacy is ongoing in larger cohorts of patients naturally exposed to
413 either seasonal (pollens) or perennial (house dust mites) allergens.

Authors contribution

KJ, JC, SL, SH, CB, PD and SCM performed experiments. NC, JB, VL, SM and CK performed experiments and contributed to manuscript writing. VBB, EN and PM contributed to study design and manuscript writing.

Acknowledgement

NC was supported by a CIFRE fellowship from ANRT (Association Nationale de la Recherche et de la Technologie).

Figure legends

Figure 1. Levels of acidic forms of FetA in pre-treatment sera from grass-pollen allergic patients correlate with SLIT clinical efficacy.

(A) 2D-gel image of serum proteins, with localization of FetA proteoforms. (B) Volumes of FetA proteoforms in spots 439 and 469 from sera of AR relative to ANR patients. Spot volumes are shown as individual results and means $\pm$ SEM in active (C) and placebo (D) groups (left panels). p-values < 0.05 (*), 0.01 (**) or 0.001 (***). Correlations of acidic-protein spot volumes with ARTSS improvement in patients from active (C) and placebo (D) groups (right panels). (E) Abundances of acidic FetA spots were assessed by 2D-DiGE before and after SLIT in sera from patients receiving placebo or active tablets.

Figure 2. Sialylation of FetA on O-linked glycans.

(A) Deconvoluted MS spectrum of FetA (A chain) purified from human serum. Mass differences corresponding to mono- or disaccharide are indicated on the spectrum. (B) 2D-gel image of a mixture of sialylated FetA (labeled in green with Cy2) and AsialoFetA (labeled in red with Cy3). (C) Composition of O-glycopeptides determined by LC-MS/MS. Yellow square = N-acetylglucosamine; yellow circle = hexose; purple diamond = N-acetylneuraminic acid (sialic acid, Neu5Ac).

Figure 3. Levels of O-linked sialylated FetA in pre-treatment sera from grass-pollen allergic patients.

O-glycopeptide abundances are expressed in arbitrary units (AU) and as means $\pm$ SEM in active (A) and placebo (B) groups. p-values < 0.05 (*) or 0.001 (***). Correlations of O-glycopeptide 3 abundance with ARTSS improvement in patients from active (A) and placebo (B) groups (right panels) after SLIT. (C) ROC analysis of OG3 abundance in serum samples from patients of the active group.

Figure 4. *In vivo* silencing of the gene encoding FetA enhances OVA-induced allergic inflammation in BALB/c mice.

(A) FetA silencing in OVA-sensitized animals was achieved by injections of FetA-specific vivo-morpholino. (B) FetA concentrations in sera are expressed, as mean values relative to D-2 values normalized to 100; $n = 3$ mice per group. p-values < 0.05 (*). (C) Airway hyperresponsiveness measured by whole body plethysmography is expressed as a Penh index (mean values $\pm$ SEM, $n = 6$ mice per group). (D) Lung resistance (Rl) was assessed by invasive plethysmography (mean values $\pm$ SEM, $n = 6$ mice per group). p-values < 0.05 (*) or < 0.0001 (****). (E) Cytokine secretion was assessed in splenocytes from animals untreated or treated with morpholinos (mean values $\pm$ SEM, $n = 6$ mice per group) p-values < 0.01 (**). (F) Identification by LC-MS/MS of sialic acid-containing O-glycopeptides associated with murine FetA. Yellow square = N-acetylglucosamine; yellow circle = hexose; clear diamond = N-glycolylneuraminic acid (Neu5Gc).

Figure 5. FetA, but not AsialoFetA, synergizes with LPS in a TLR4-dependent pathway to enhance the proallergic profile of human MoDCs.

(A and B) TLR4 activation was measured in hTLR4 HEK-293 reporting cells. p-values <0.05 (#). (C) Expression of co-stimulatory molecules. (D) Cytokine production and (E) Gene expression by MoDCs cultured in presence of either FetA or AsialoFetA. Data are shown as means $\pm$ SEM (n = 6 to 8 donors tested) (C-E). Cytokine production was measured in immature MoDCs cultured in presence of either FetA or AsialoFetA plus either a 5-grass pollen (F), or *D. farinae* allergen extract (G) Results are presented as means $\pm$ SEM (n = 6 donors tested). p-values < 0.05 (*) or 0.0001 (****).

Figure 6. FetA and AsialoFetA bind LPS, but do not influence its physical binding to TLR4-MD2.

Surface plasmon resonance analyses of the interaction between injected LPS and FetA (A) or AsialoFetA (B) immobilized on CM5 sensorchips. Sensorgrams obtained following injection of either LPS (C), a combination of LPS with FetA (D) or LPS with AsialoFetA (E) over TLR4-MD2 immobilized on a CM5 sensor chip. Sensorgrams are represented as response units (RUs) at specified times in seconds (s).

References

1. Cox L, Nelson H, Lockey R, Calabria C, Chacko T, Finegold I, et al. Allergen immunotherapy: a practice parameter third update. *J Allergy Clin Immunol* 2011; 127:S1-55.
2. Calderon MA, Casale TB, Togias A, Bousquet J, Durham SR, Demoly P. Allergen-specific immunotherapy for respiratory allergies: from meta-analysis to registration and beyond. *J Allergy Clin Immunol* 2011; 127:30-8.
3. Canonica GW, Cox L, Pawankar R, Baena-Cagnani CE, Blaiss M, Bonini S, et al. Sublingual immunotherapy: World Allergy Organization position paper 2013 update. *World Allergy Organ J* 2014; 7:6.
4. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001; 69:89-95.
5. Moingeon P. Update on immune mechanisms associated with sublingual immunotherapy: practical implications for the clinician. *J Allergy Clin Immunol Pract* 2013; 1:228-41.
6. Shamji MH, Ljorring C, Wurtzen PA. Predictive biomarkers of clinical efficacy of allergen-specific immunotherapy: how to proceed. *Immunotherapy* 2013; 5:203-6.
7. Willis JC, Lord GM. Immune biomarkers: the promises and pitfalls of personalized medicine. *Nat Rev Immunol* 2015; 15:323-9.
8. Moingeon P. Biomarkers for Allergen Immunotherapy: A "Panoromic" View. *Immunol Allergy Clin North Am* 2016; 36:161-79.
9. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol* 2011; 127:18-27.
10. Shamji MH, Durham SR. Mechanisms of immunotherapy to aeroallergens. *Clin Exp Allergy* 2011; 41:1235-46.
11. Akdis M, Akdis CA. Mechanisms of allergen-specific immunotherapy: Multiple suppressor factors at work in immune tolerance to allergens. *J Allergy Clin Immunol* 2014; 133:621-31.
12. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol* 2007; 120:707-13.
13. Shamji MH, Ljorring C, Francis JN, Calderon MA, Larche M, Kimber I, et al. Functional rather than immunoreactive levels of IgG4 correlate closely with clinical response to grass pollen immunotherapy. *Allergy* 2012; 67:217-26.
14. Zimmer A, Bouley J, Le Mignon M, Pliquet E, Horiot S, Turfkruyer M, et al. A regulatory dendritic cell signature correlates with the clinical efficacy of allergen-specific sublingual immunotherapy. *J Allergy Clin Immunol* 2012; 129:1020-30.
15. Gueguen C, Bouley J, Moussu H, Luce S, Duchateau M, Chamot-Rooke J, et al. Changes in markers associated with dendritic cells driving the differentiation of either TH2 cells or regulatory T cells correlate with clinical benefit during allergen immunotherapy. *J Allergy Clin Immunol* 2016; 137:545-58.
16. Bufe A, Ziegler-Kirbach E, Stoeckmann E, Heidemann P, Gehlhar K, Holland-Letz T, et al. Efficacy of sublingual swallow immunotherapy in children with severe grass pollen allergic symptoms: a double-blind placebo-controlled study. *Allergy* 2004; 59:498-504.
17. Howarth P, Malling HJ, Molimard M, Devillier P. Analysis of allergen immunotherapy studies shows increased clinical efficacy in highly symptomatic patients. *Allergy* 2012; 67:321-7.

18. Van Overtvelt L, Baron-Bodo V, Horiot S, Moussu H, Ricarte C, Horak F, et al. Changes in basophil activation during grass-pollen sublingual immunotherapy do not correlate with clinical efficacy. *Allergy* 2011; 66:1530-7.
19. Di Lorenzo G, Mansueto P, Pacor ML, Rizzo M, Castello F, Martinelli N, et al. Evaluation of serum s-IgE/total IgE ratio in predicting clinical response to allergen-specific immunotherapy. *J Allergy Clin Immunol* 2009; 123:1103-10.
20. Ciprandi G, Silvestri M. Serum specific IgE: a biomarker of response to allergen immunotherapy. *J Investig Allergol Clin Immunol* 2014; 24:35-9.
21. Cox LS, Casale TB, Nayak AS, Bernstein DI, Creticos PS, Ambrosine L, et al. Clinical efficacy of 300IR 5-grass pollen sublingual tablet in a US study: the importance of allergen-specific serum IgE. *J Allergy Clin Immunol* 2012; 130:1327-34.
22. Fujimura T, Yonekura S, Horiguchi S, Taniguchi Y, Saito A, Yasueda H, et al. Increase of regulatory T cells and the ratio of specific IgE to total IgE are candidates for response monitoring or prognostic biomarkers in 2-year sublingual immunotherapy (SLIT) for Japanese cedar pollinosis. *Clin Immunol* 2011; 139:65-74.
23. Horak F, Zieglmayer P, Zieglmayer R, Lemell P, Devillier P, Montagut A, et al. Early onset of action of a 5-grass-pollen 300-IR sublingual immunotherapy tablet evaluated in an allergen challenge chamber. *J Allergy Clin Immunol* 2009; 124:471-7.
24. Huang J, Lee H, Zivkovic AM, Smilowitz JT, Rivera N, German JB, et al. Glycomic analysis of high density lipoprotein shows a highly sialylated particle. *J Proteome Res* 2014; 13:681-91.
25. Mori K, Emoto M, Inaba M. Fetuin-A: a multifunctional protein. *Recent Pat Endocr Metab Immune Drug Discov* 2011; 5:124-46.
26. Heinrichsdorff J, Olefsky JM. Fetuin-A: the missing link in lipid-induced inflammation. *Nat Med* 2012; 18:1182-3.
27. Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, et al. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med* 2012; 18:1279-85.
28. Stefan N, Haring HU. Circulating fetuin-A and free fatty acids interact to predict insulin resistance in humans. *Nat Med* 2013; 19:394-5.
29. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* 2011; 32:402-11.
30. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009; 15:410-6.
31. Mittag D, Varese N, Scholzen A, Mansell A, Barker G, Rice G, et al. TLR ligands of ryegrass pollen microbial contaminants enhance Th1 and Th2 responses and decrease induction of Foxp3(hi) regulatory T cells. *Eur J Immunol* 2013; 43:723-33.
32. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009; 457:585-8.
33. Coats SR, Pham TT, Bainbridge BW, Reife RA, Darveau RP. MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize *Escherichia coli* lipopolysaccharide at the TLR4 signaling complex. *J Immunol* 2005; 175:4490-8.
34. Eifan AO, Shamji MH, Durham SR. Long-term clinical and immunological effects of allergen immunotherapy. *Curr Opin Allergy Clin Immunol* 2011; 11:586-93.

35. Weberschock T, Schaefer I, Heigel H, Valesky E, Augustin M, Schmitt J. Use of specific immunotherapy--a survey of 15 164 employed persons in Germany. *J Dtsch Dermatol Ges* 2014; 12:341-5.
36. Halim A, Nilsson J, Ruetschi U, Hesse C, Larson G. Human urinary glycoproteomics; attachment site specific analysis of N- and O-linked glycosylations by CID and ECD. *Mol Cell Proteomics* 2012; 11:M111 013649.
37. Sato H, Kazama JJ, Wada Y, Kuroda T, Narita I, Gejyo F, et al. Decreased levels of circulating alpha2-Heremans-Schmid glycoprotein/Fetuin-A (AHSG) in patients with rheumatoid arthritis. *Intern Med* 2007; 46:1685-91.
38. Saroha A, Kumar S, Chatterjee BP, Das HR. Jacalin bound plasma O-glycoproteome and reduced sialylation of alpha 2-HS glycoprotein (A2HSG) in rheumatoid arthritis patients. *PLoS One* 2012; 7:e46374.
39. Jahn-Dechent W, Heiss A, Schafer C, Ketteler M. Fetuin-A regulation of calcified matrix metabolism. *Circ Res* 2011; 108:1494-509.
40. Herrmann M, Kinkeldey A, Jahn-Dechent W. Fetuin-A function in systemic mineral metabolism. *Trends Cardiovasc Med* 2012; 22:197-201.
41. Goustin AS, Derar N, Abou-Samra AB. Ahsg-fetuin blocks the metabolic arm of insulin action through its interaction with the 95-kD beta-subunit of the insulin receptor. *Cell Signal* 2013; 25:981-8.
42. Wang H, Li W, Zhu S, Li J, D'Amore J, Ward MF, et al. Peripheral administration of fetuin-A attenuates early cerebral ischemic injury in rats. *J Cereb Blood Flow Metab* 2010; 30:493-504.
43. Wang H, Sama AE. Anti-inflammatory role of fetuin-A in injury and infection. *Curr Mol Med* 2012; 12:625-33.
44. Ketteler M, Bongartz P, Westenfeld R, Wildberger JE, Mahnen AH, Bohm R, et al. Association of low fetuin-A (AHSG) concentrations in serum with cardiovascular mortality in patients on dialysis: a cross-sectional study. *Lancet* 2003; 361:827-33.
45. Metry G, Stenvinkel P, Qureshi AR, Carrero JJ, Yilmaz MI, Barany P, et al. Low serum fetuin-A concentration predicts poor outcome only in the presence of inflammation in prevalent haemodialysis patients. *Eur J Clin Invest* 2008; 38:804-11.
46. Stenvinkel P, Wang K, Qureshi AR, Axelsson J, Pecoits-Filho R, Gao P, et al. Low fetuin-A levels are associated with cardiovascular death: Impact of variations in the gene encoding fetuin. *Kidney Int* 2005; 67:2383-92.
47. Brix JM, Stingl H, Hollerl F, Schernthaner GH, Kopp HP, Schernthaner G. Elevated Fetuin-A concentrations in morbid obesity decrease after dramatic weight loss. *J Clin Endocrinol Metab* 2010; 95:4877-81.
48. Stefan N, Fritsche A, Weikert C, Boeing H, Joost HG, Haring HU, et al. Plasma fetuin-A levels and the risk of type 2 diabetes. *Diabetes* 2008; 57:2762-7.
49. Tuttolomondo A, Di Raimondo D, Di Sciacca R, Casuccio A, Bivona G, Bellia C, et al. Fetuin-A and CD40 L plasma levels in acute ischemic stroke: differences in relation to TOAST subtype and correlation with clinical and laboratory variables. *Atherosclerosis* 2010; 208:290-6.
50. Barboza R, Camara NO, Gomes E, Sa-Nunes A, Florsheim E, Mirotti L, et al. Endotoxin Exposure during Sensitization to Allergens Shifts TH2 Immunity Towards a TH17-Mediated Airway Neutrophilic Inflammation: Role of TLR4 and TLR2. *PLoS One* 2013; 8:e67115.
51. Karamessinis PM, Malamitsi-Puchner A, Boutsikou T, Makridakis M, Vougas K, Fountoulakis M, et al. Marked defects in the expression and glycosylation of alpha2-HS glycoprotein/fetuin-A in plasma from neonates with intrauterine growth

628 restriction: proteomics screening and potential clinical implications. Mol Cell
629 Proteomics 2008; 7:591-9.

630

ACCEPTED MANUSCRIPT

Figure 1

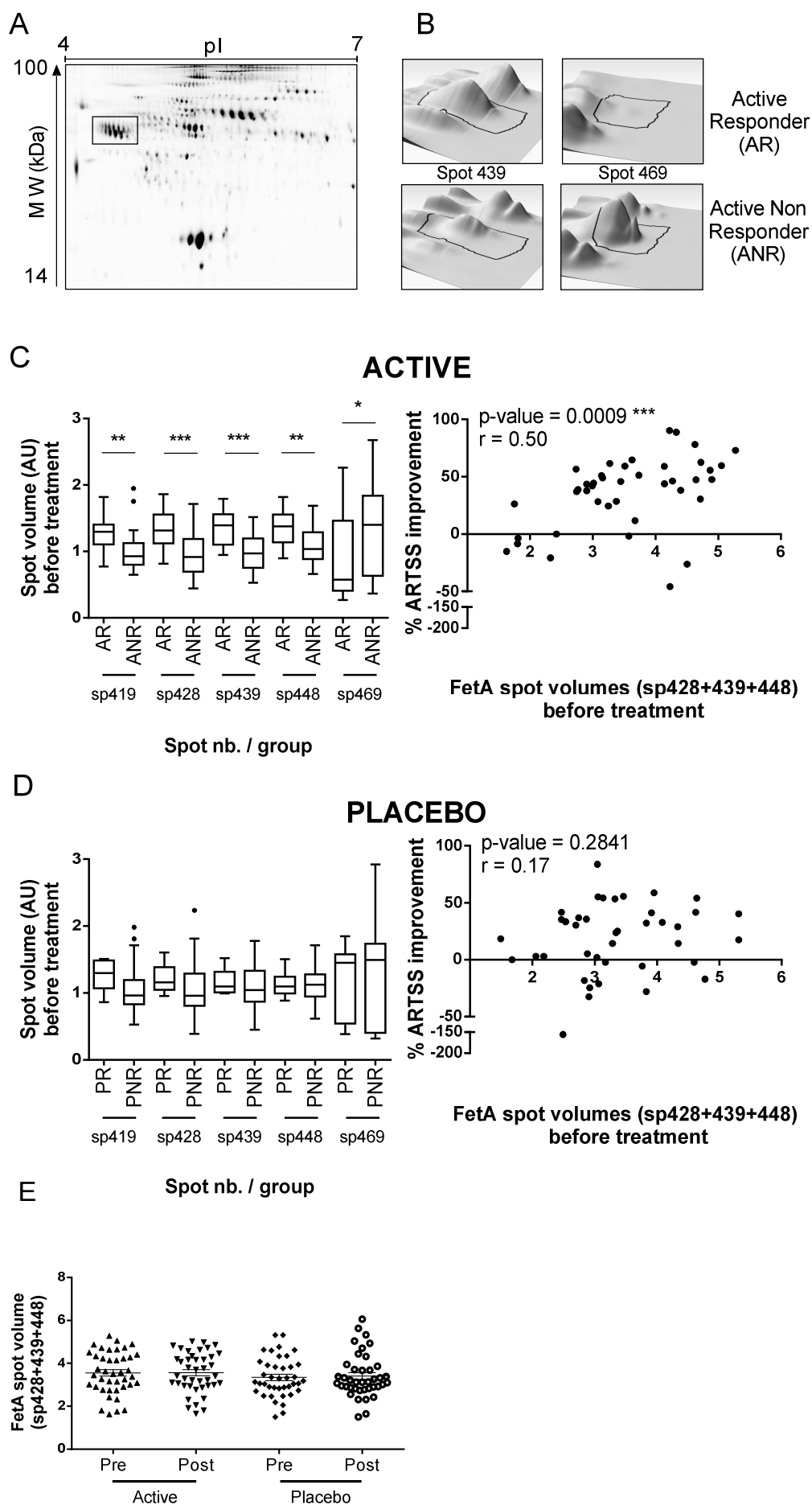


Figure 2

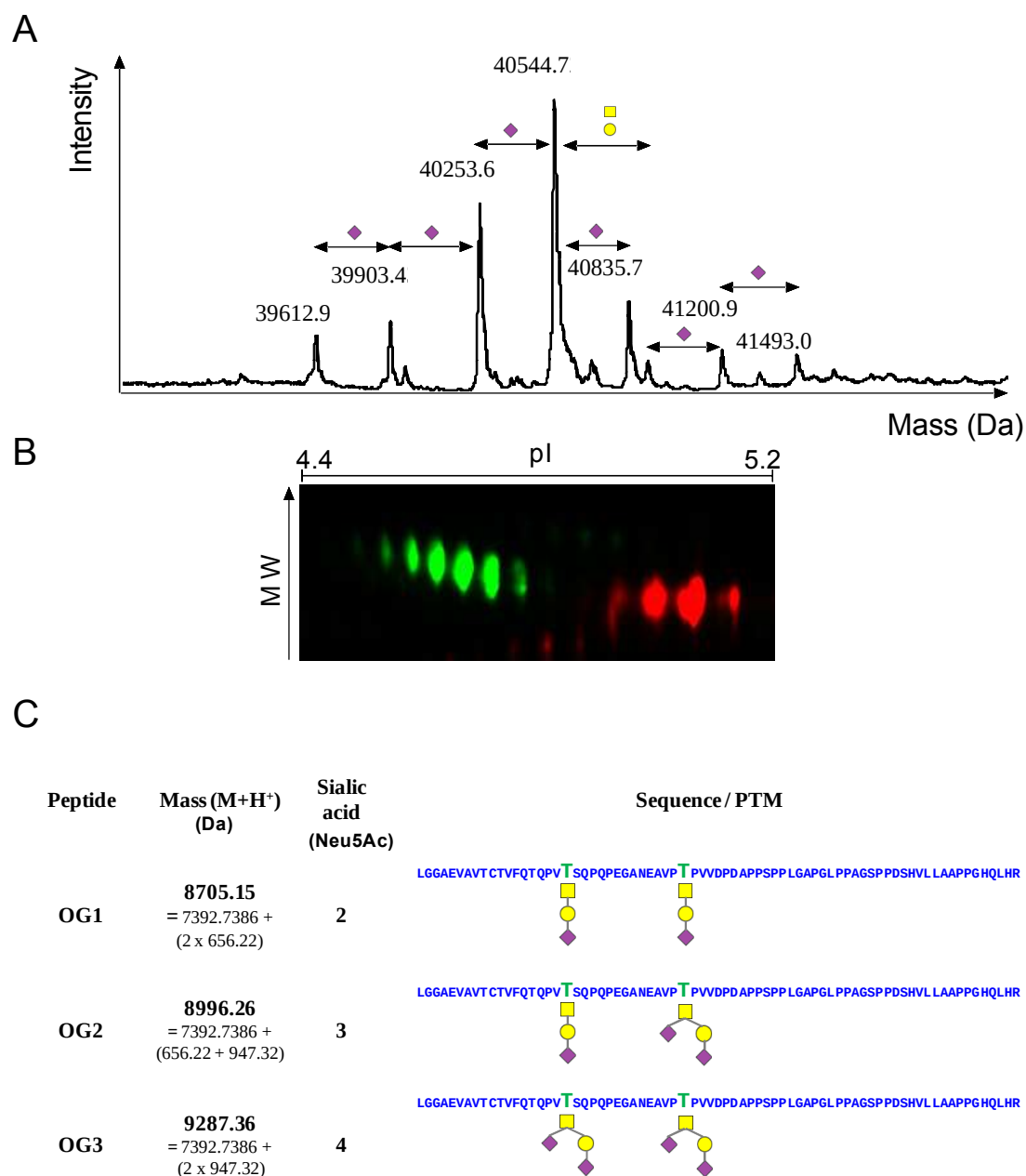


Figure 3

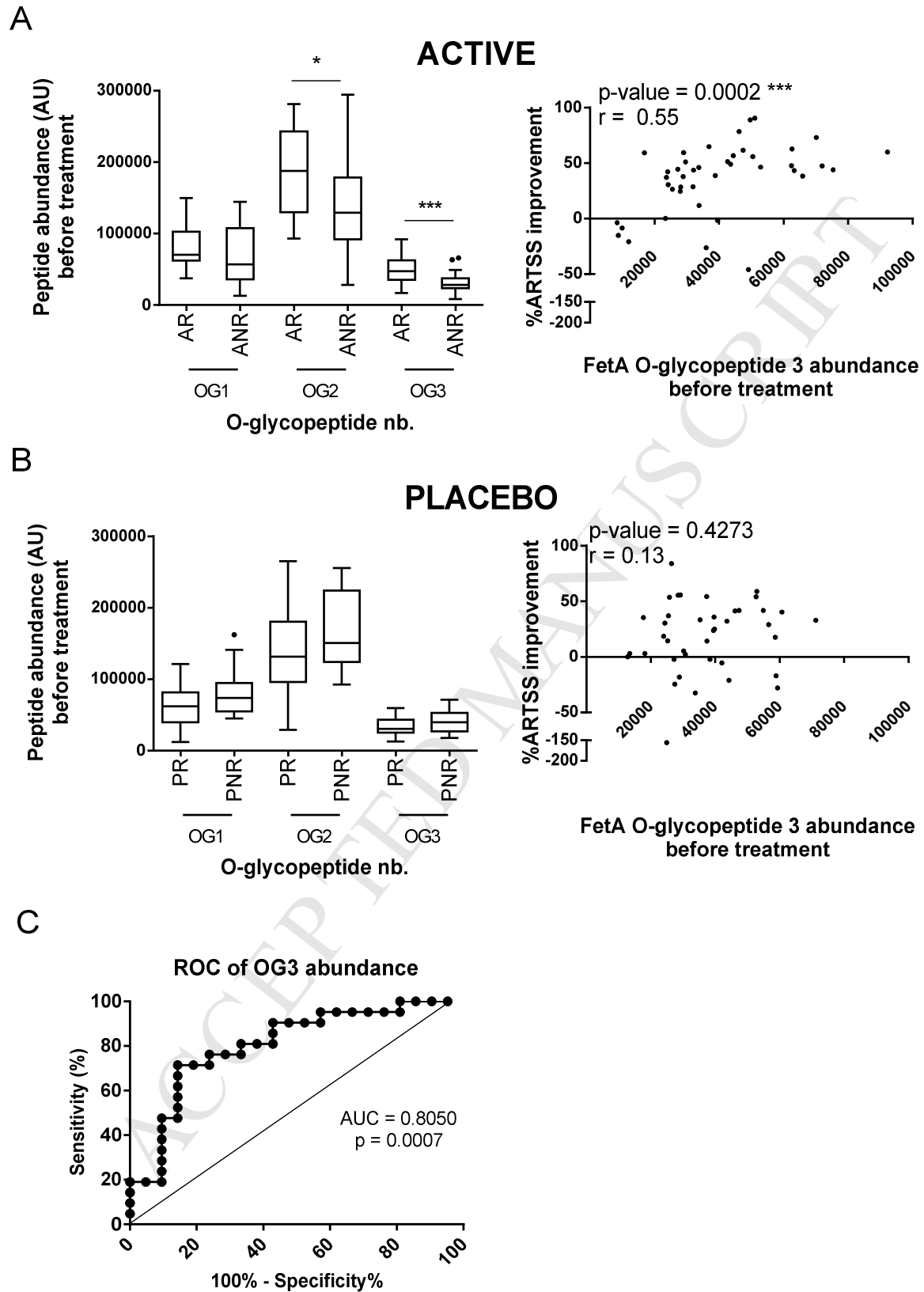
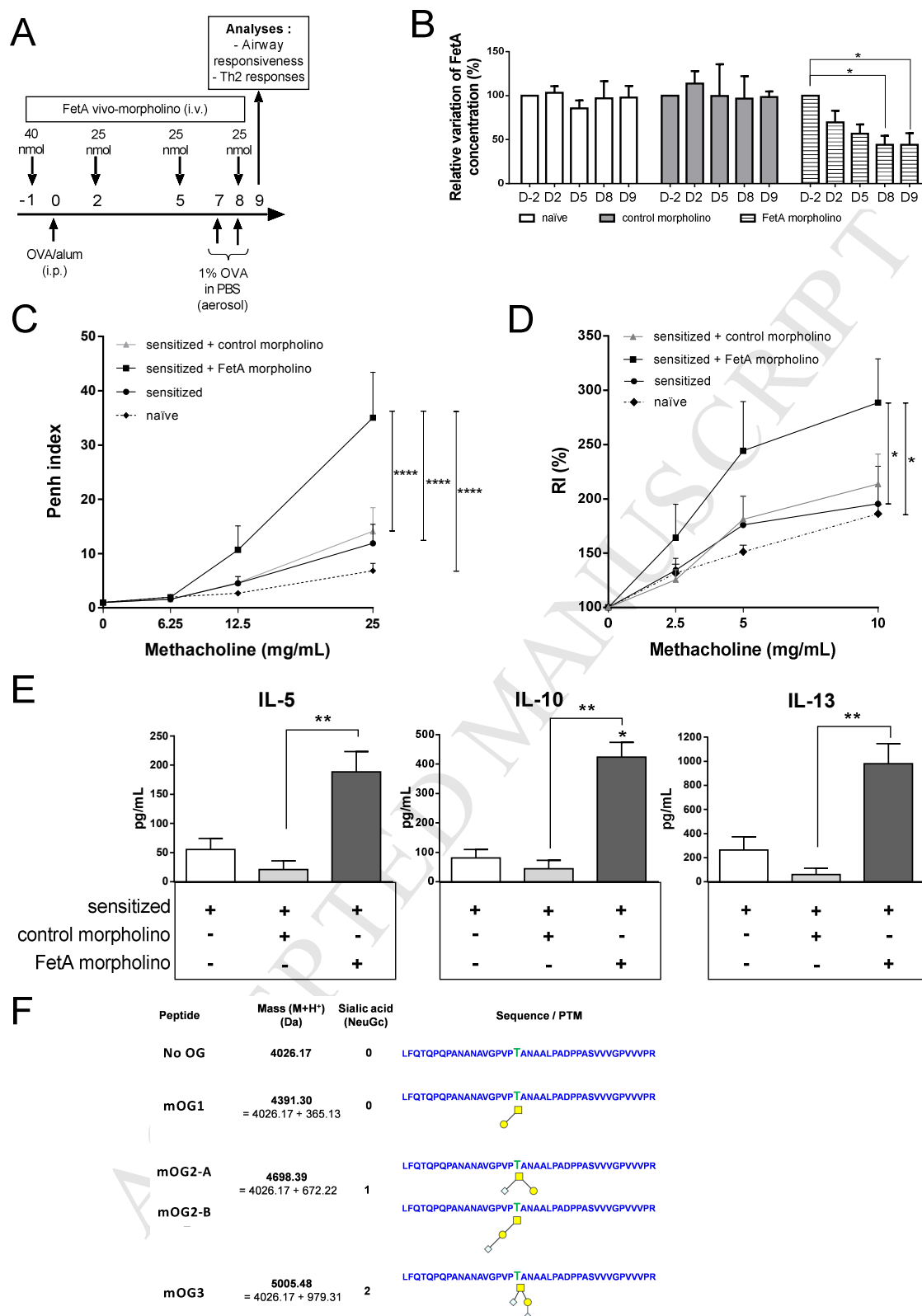


Figure 4



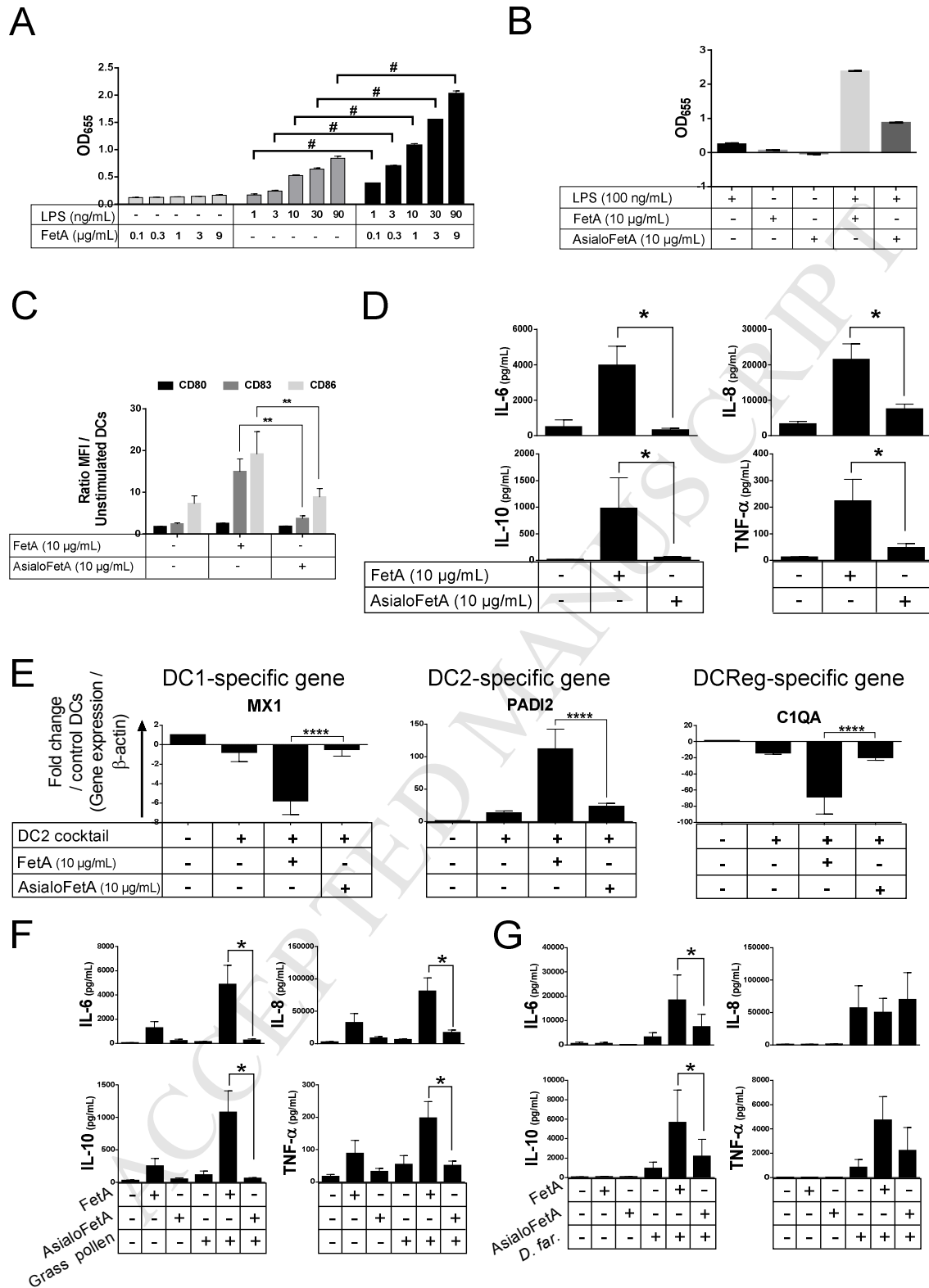
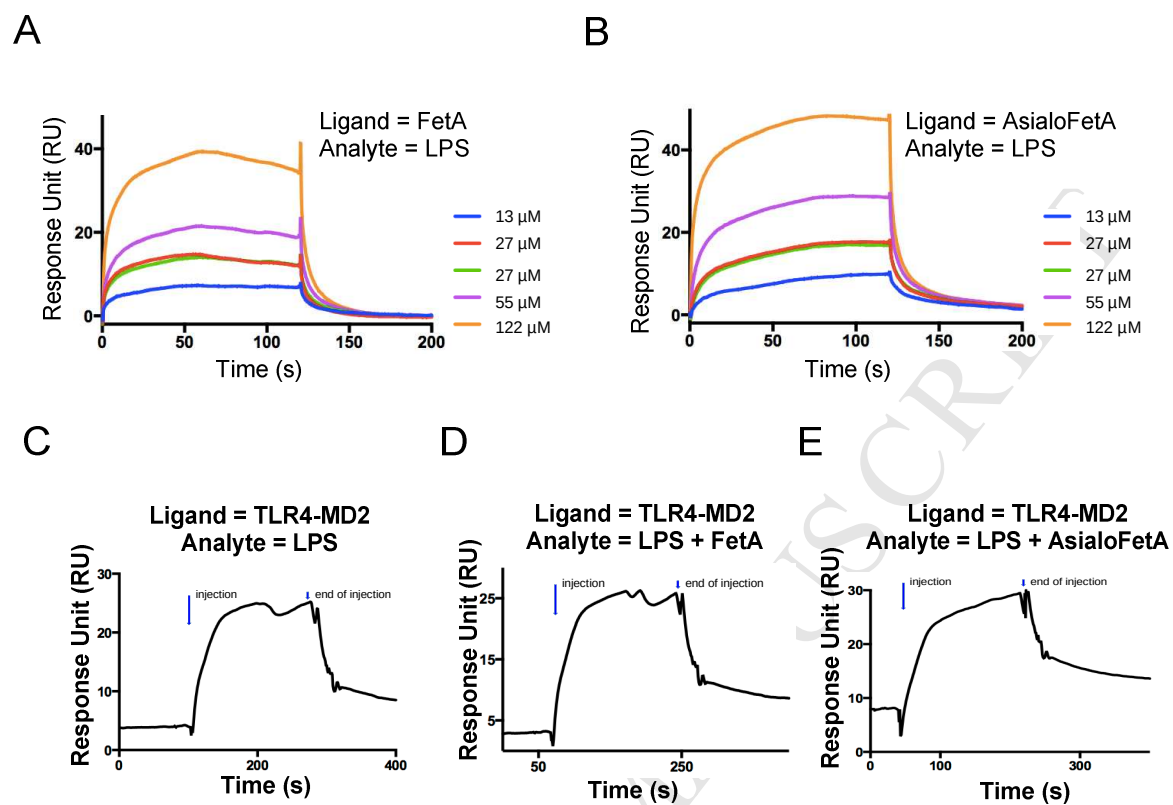


Figure 6



Supplementary Methods*Study approval*

The VO56.07 clinical study was carried out in accordance with international good clinical practice and was approved by both an independent ethics committee and regulatory authorities (ClinicalTrials.gov identifier: NCT00619827). All patients provided written informed consent before study entry. For mouse studies, international standards were applied for animal handling, and animal protocols were validated by an internal ethics committee.

Serum depletion

Serum samples (340 μ L each) from grass pollen allergic patients were depleted from the 6 most abundant proteins using a human Multiple Affinity Removal System (MARS) Human 6 (Hu-6HC column, 10 x 100 mm, Agilent Technologies, Palo Alto, USA) and an Ultimate 3000 HPLC apparatus (Thermo Scientific, Waltham, USA). Flow-through proteins were collected and concentrated according to the manufacturer's instructions. Following depletion, samples were stored at -80°C until analysis. The run-to-run reproducibility of depletion was confirmed by chromatography and SDS-PAGE analyses under reducing conditions, using 4-12% NuPAGE gels according to the manufacturer's instructions (Thermo Scientific).

2-dimensional differential gel electrophoresis (2D-DiGE)-based proteomics

Protein samples from depleted sera were precipitated using a 2D clean-up kit according to GE Healthcare's (Fairfield, USA) protocol, with precipitates resolubilized in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris pH 8.8 (all obtained from Sigma-Aldrich, Saint-Louis, USA) and stored at -80°C. Protein concentrations were determined using the

Bradford assay (BioRad, Hercules, USA) and 50 µg of depleted serum proteins were labeled with 400 pmol of either Cy2, Cy3, or Cy5 dyes (GE Healthcare), as described in the instruction manual. A dye-swapping scheme was used to preclude any specific dye-labeling artifact. The Cy2-labelled internal standard was obtained by pooling equal amounts (25 µg) of proteins from all 82 depleted sera. Protein samples labeled with Cy2, Cy3 and Cy5 dyes were mixed and diluted with a rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT and 0.5% IPG, under a final volume of 450 µL. Proteins (150 µg) were then applied to 24 cm-long immobilized pH 4–7 gradient strips (GE Healthcare) according to the manufacturer's instructions. Isoelectric focusing (IEF) was performed for a total of 74 kVh using an IPGPhor 3 electrophoresis unit (GE Healthcare) cooled to 18°C. Strips were then equilibrated in urea-containing buffer, and placed on top of pre-cast SDS-12.5% polyacrylamide gels (GE Healthcare). Electrophoresis was carried out at 0.5 W for 1 h followed by 1 W for 16 h (using an Ettan DALT Twelve Electrophoresis System, GE Healthcare). DiGE gels were scanned with an Ettan DiGE Imager (GE Healthcare) according to the manufacturer's instructions. Differentially expressed spots were determined by image analysis with the SameSpots program (Nonlinear Dynamics, Newcastle upon Tyne, UK, v4.1) and selected for automatic spot picking (EXquestTM Spot Cutter, BioRad). Preparative gels stained with Sypro Ruby (Thermo Scientific) were used for spot picking and protein identification by tandem mass spectrometry (MS/MS).

To this aim, gel plugs were digested with trypsin (from porcine pancreas, Sigma-Aldrich) and the resulting peptides were separated by reversed-phase chromatography using an Ultimate 3000 RS-nanoLC system (Thermo Scientific) coupled to a high-resolution Maxis 4G ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Peptides were injected and trapped on an Acclaim® PepMap100 column (100 µm x 2 cm; C₁₈, 5 µm particles and pore size at 100 Å, Thermo Scientific), followed by separation using a C₁₈ column (Acclaim®

PepMap RSLC 75 μm ID, 15 cm, 2 μm particles, and pore size at 100 $\text{\AA}$, Thermo Scientific). NanoLC-MS/MS data were analyzed using an in-house Mascot server (Matrix Science Ltd, London, UK, v2.3) or PEAKS software (Bioinformatics Solutions Inc., Waterloo, Canada, v7) to search the UniprotKB/TrEMBL and nonredundant National Center for Biotechnology Information (nrNCBI) databases (downloaded Dec 2013), assuming tryptic digestion. Precursor and fragment masses were searched with a mass tolerance of 8 ppm and 0.05 Da, respectively. The search included fixed modification of carbamidomethyl (CAM) cysteine. The minimal peptide length was set to 6 amino acids, with a single miscleavage allowed. Peptide identifications were accepted when established with a probability $\geq 95\%$, as specified by Mascot or PEAKS software.

Label-free mass spectrometry-based proteomics

Fifty micrograms of depleted serum proteins were solubilized in a buffer containing 8 M urea and 75 mM Tris pH 8.5, reduced and alkylated with 20 mM DTT and 50 mM iodoacetamide (IAA), respectively, and digested with trypsin (1:25 w/w) at 37°C overnight. Peptides were then acidified with 2.5% formic acid (FA) and analyzed by LC-MS/MS using the Ultimate 3000 RSLC system (Thermo Scientific) coupled to the ESI-Qq-TOF mass spectrometer. Five micrograms of tryptic peptides were injected on an Acquity C₁₈ BEH130 column (10 mm ID, 10 cm, 1.7 μm particles, Waters, Milford, USA) equilibrated at 40°C with 95% solvent A (0.15% FA) and 5% solvent B (100% ACN, 0.15% FA) at a flow rate of 0.4 mL/min. Peptide separation was performed at a flow rate of 0.4 mL/min with a linear gradient obtained using 5 to 40% solvent B over 30 min. Ion intensities recorded in LC-MS data were analyzed using the Progenesis LC-MS v4.1 software (Nonlinear Dynamics) to provide reliable measurements of peptide abundance across samples. Lock mass calibration and peptide detection were performed with peptide intensity > 1000, peptide abundance > 2000 and peptide charge

between 2+ and 12+. Data were then normalized by the “normalize to all features” method and peptides differentially represented in samples obtained from the four patient subgroups (*i.e.* ARs, ANRs, PRs and PNRs) were identified. LC-MS/MS data were analyzed using an in-house Mascot server against the Swiss-Prot database, taxonomy *Homo sapiens*, assuming tryptic digestion, as described above.

Characterization of human FetA and AsialoFetA

FetA was purified from pooled sera obtained from AB⁺ individuals (Lonza, Walkersville, USA), diluted 1/10 v/v in PBS 1X. Affinity chromatography was performed using a human FetA-specific murine monoclonal antibody (clone 162919, R&D Systems, Minneapolis, USA), coupled to a 5 mL Hitrap affinity column pre-equilibrated in binding buffer (PBS 1X pH 7.4). After loading samples at a flow rate of 0.8 mL/min, the column was washed with 8 volumes of binding buffer until the optical density (OD₂₁₄) dropped below 5% of its maximum value. FetA was eluted by running 7 volumes of 50 mM Glycin-HCl pH 3 at a flow rate of 1 mL/min, followed by neutralization with 1 M Tris-HCl pH 9. Subsequently, samples were diafiltered and concentrated using an Amicon ultra-15 mL membrane with a cutoff of 10 kDa (Millipore, Billerica, USA), prior to SDS-PAGE (4-12% NuPAGE gel, Thermo Scientific) and western blotting analysis, using an anti-FetA monoclonal antibody (R&D Systems) followed by an anti-mouse sheep IgG-peroxidase antibody (Sigma-Aldrich) for revealing.

For desialylation, 31.2 µg of native purified FetA were mixed with 10 µL of 5X Reaction Buffer and 1 µL of α-(2→3,6,8,9)-Neuraminidase (EDEGLY kit, Sigma-Aldrich), to reach a final enzymatic activity of 0.109 U/mL. The mixture was incubated overnight at 37°C, and complete desialylation was confirmed by SDS-PAGE (4-12% NuPAGE gel, Thermo Scientific) and MS characterization. AsialoFetA samples were run on Detoxi-Gel Endotoxin Removing Columns (Thermo Scientific), according to the manufacturer's instructions. Levels of endotoxin in FetA and AsialoFetA samples, controlled by a chromogenic Limulus Amebocyte Lysate (LAL) assay (LAL Chromogenic Endotoxin Quantitation Kit, Thermo Scientific) were always below 0.0002 EU/mg.

Measurements of the average mass (M_{av}) of intact purified FetA or AsialoFetA were performed on a Maxis ESI-Qq-TOF mass spectrometer coupled to an Ultimate 3000 RSLC

system (Thermo Scientific). Proteins were denatured and reduced in a buffer containing 8 M urea, 75 mM Tris pH 8.5, 20 mM DTT for 20 min and acidified by 2.5% FA. Protein samples were then desalted and concentrated for 4 min onto a Acquity C₄ BEH300 column (10 mm ID, 10 cm, 1.7 μ m particles, Waters) equilibrated at 70°C with 95% solvent A (0.15% FA) and 5% solvent B (100% ACN, 0.15% FA) at a flow rate of 0.4 mL/min. Proteins were eluted directly into the mass spectrometer with a linear gradient from 5 to 60% solvent B over 30 min. Mass spectra were deconvoluted using MaxEnt software, and the precise Mav of proteins was determined with the Data Analysis software (Bruker Daltonics, v4.2).

Post-translational modifications (PTMs) of purified FetA or AsialoFetA were determined by nanoLC-MS/MS as described above. Purified proteins were solubilized in a buffer containing 8 M urea, 75 mM Tris pH 8.5 and 5 mM TCEP. Proteins were then alkylated with 10 mM IAA for 20 min and digested in solution with trypsin (1:50 w/w) for 3 h at 37°C in presence of 0.02% ProteaseMax surfactant (Promega, Madison, USA). Subsequently, 2.5% FA was added to the mixture to quench enzymatic activity and peptides were stored at -80°C until analysis. Peptide samples were then spun at 18,000 g and run on a C18 column (Acclaim® PepMap RSLC 75 μ m ID, 25 cm, 2 μ m particles, and pore size at 100 Å, Thermo Scientific). N- and O-glycopeptides were manually characterized, based on the presence of glycan-specific oxonium ion fragments, using the Data Analysis software.

Characterization of murine FetA

Murine FetA was enriched from serum from BALB/c mice by immunocapture using an anti-murine FetA monoclonal antibody (clone DY1563, R&D Systems) covalently linked to magnetic beads (Dynabeads M-280, Thermo scientific). This bead suspension was incubated for 2 hours with sera from BALB/c mice diluted in PBS (1:1, v/v), washed twice with PBS and proteins were eluted with a 50% ACN, 0.1% trifluoroacetic acid (TFA) solution. FetA

immunoprecipitates were dried by vacuum centrifugation and resuspended in 50 mM ammonium bicarbonate buffer, pH 8.0. Following reduction with 45 mM DTT at 60°C for 30 min, and alkylation with 100 mM IAA at room temperature for 45 min, samples were incubated overnight at 37°C with 1 µg trypsin. After quenching with formic acid (0.5% v/v final), samples were injected into a C18 column (Acclaim® PepMap RSLC 75 µm ID, 25 cm, 2 µm particles, and pore size at 100 Å, Thermo Scientific). The column effluent was directly introduced into the electrospray source of an ImpactHD ESI-Qq-TOF mass spectrometer (Bruker Daltonics). Characterization of the glycosylation pattern was performed manually using the Data Analysis software.

Evaluation of immunomodulatory properties of FetA and AsialoFetA

In vivo silencing of the FetA gene was performed in female BALB/c mice (6 to 8 weeks of age, Charles River, Wilmington, US) to assess its impact on allergic inflammation. Mice were sensitized with an intraperitoneal injection of 10 µg ovalbumin (OVA, Sigma-Aldrich) adsorbed on 2 mg Al(OH)₃, followed by a 20-minute aerosol challenge at days 7 and 8 using 50 µg OVA per mouse. Alternatively, naive mice were sensitized by the intraperitoneal route with a timothy grass pollen extract (using a dose containing 10 µg of Phl p 5, Stallergenes SA, Antony, France) mixed with alum followed by two intranasal challenges with the timothy grass pollen extract in PBS (equivalent to 10 µg of Phl p 5), performed on days 7 and 8 after the first immunization. In both experimental settings, FetA silencing was achieved by 4 intravenous injections of a specific vivo-morpholino (5'-AAGACCAGGGACTTCATGGTTGCTC-3', Gene Tools, Philomath, USA), using 40 nM one day prior to sensitization and 25 nM at days 2, 5 and 7. A standard irrelevant vivo-morpholino sequence was used in parallel for mock treatment of control animals²³. Measurements of airway hyperresponsiveness (AHR) were performed by whole body

plethysmography using a Buxco (Wilmington, USA) apparatus after exposure to increasing doses (0 to 50 mg/mL) of nebulized methacholine, and determination of enhanced pause (Penh) values²⁴. Lung resistance was measured using a FinePointe RC system (Buxco), and mice were subsequently sacrificed. Serum FetA levels were assessed by ELISA (R&D Systems) on retro-orbital bleeds at 4 time points during sensitization and after sacrifice. Splenocytes were stimulated with either 100 µg/mL OVA or medium for 72 hours at 37°C under a 5% CO₂ atmosphere and cell supernatants were collected to measure IL-5, IL-10, IL-13 and IFN-γ concentrations using a Luminex assay (Millipore).

Monocytes isolated from PBMCs of healthy volunteers with CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were cultured in presence of GM-CSF and IL-4 (Miltenyi Biotec) for 6 days. Dendritic cell (DC) differentiation was confirmed by flow cytometry based on the loss of CD14 expression and the upregulation of CD1a and CD11c surface expression. Immature monocyte-derived DCS (MoDCs) or MoDCs polarized towards a DC2 pattern (using a mixture of histamine, IL-25, IL-33, TSLP and 10 ng/ml LPS) were cultured in serum-free medium²⁵ (CellGro DC medium, Cellgenix, Freiburg, Germany) for 24 hours, in presence of either medium alone, or medium supplemented with purified native FetA (10 µg/mL), AsialoFetA (10 µg/mL), a grass pollen or *Dermatophagoides farinae* allergen extract (20 or 1 µg/mL, respectively, Stallergenes SA), or combinations of these reagents. The TLR4 signaling pathway was blocked by adding LPS from *Rhodobacter sphaeroides* (LPS-RS, 10 µg/mL, Invivogen) to the cultures for 30 minutes. Supernatants were harvested to measure cytokine (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α) production using a Luminex assay (Milliplex, Millipore). Surface expression of maturation markers (CD80, CD83 and CD86) was assessed on DCs by flow cytometry using specific antibodies (BD Biosciences, San Jose, US). Total RNA (RNeasy Mini Kit, Qiagen, Venlo, Netherlands) was isolated to evaluate the expression of C1QA, MX1, and PADI2 genes by

real-time PCR¹⁵ using β -actin as an endogenous reference gene, and the following primers, respectively, all from Thermo Scientific: Hs00381122_m1, Hs00895608_m1, and Hs00247108_m1.

Analysis of LPS–FetA–TLR4 interactions

The interaction between FetA and TLR4 was analyzed using HEK-293 epithelial cells expressing the human TLR4 receptor together with a reporter gene driven by an NF κ B inducible promoter encoding an alkaline phosphatase secreted following TLR activation. hTLR4 HEK-293 cells were incubated for 18 hours with either ultra-pure LPS-EB from *Escherichia coli* (100 ng/mL, Invivogen, Toulouse, France), purified native FetA (10 μ g/ml), AsialoFetA (10 μ g/ml) or combinations of these reagents, and TLR activation was then quantified by measuring optical densities (OD) of culture supernatants at 655 nm.

Surface plasmon resonance experiments were performed on a Biacore T200 instrument (GE Healthcare) using HC200m (CM5 type) sensor chips according to the manufacturer's instructions. Briefly, proteins were immobilized on the HC200m sensor chip by the amine-coupling method, to reach 1500 and 2500 Response Units (RU) of immobilized FetA and AsialoFetA, respectively. Blocking of the reference flow cell was performed by activation followed by immediate quenching, as for the active flow cell. A running buffer containing 10 mM Hepes pH 7.4, 150 mM NaCl and Tween (0.05% v/v) was used in all experiments, using a flow rate of 10 to 30 μ L/min. LPS was injected at various concentrations (13–122 μ M) over the FetA- or AsialoFetA-immobilized and reference flow cells. Additional studies were performed with 100 μ M LPS, or a combination of 100 μ M LPS and 300 nM FetA or AsialoFetA over a CM5 sensor chip with immobilized TLR4-MD-2. Sensorgrams were obtained by measuring the response in the active flow cell after subtracting the signal obtained in the reference cell.

Supplementary Table 1. Workflow applied for quantitative proteomic studies

Steps in chronological order	Methods	Samples tested
1) Detection of regulated proteins	2D-DiGE	20 depleted sera
2) Verification of regulated proteins	2D-DiGE	20 depleted sera
3) Confirmation of regulated proteins	2D-DiGE	82 depleted sera
4) Detection of regulated proteins	Shotgun label-free nLC-MS/MS	82 depleted sera
5) Identification of proteins detected by 2D-DiGE	nLC-MS/MS	52 protein spots
6) Confirmation of regulated proteins	WB, ELISA	82 sera
7) Characterization of glyco-proteins detected by 2D-DiGE	nLC-MS/MS	5 protein spots
8) Quantification of glycoforms of interest	Targeted LC-MS	82 depleted sera

Supplementary Tables 2

Supplementary Table 2: MS characterization of Fetuin A spots identified by 2D-DIGE.

Measured (meas.). Peptide charge state (z). No. of miscleavages (P). Range, numbering according to the pro-protein sequence. The search included cysteine carbamidomethylation (CAM) as a fixed modification.

Spot No./Picking No.	LC-MS/MS identification data							Modification (Carbamidomethylation C:)
	Accession	No. of identified peptides	m/z meas.	z	? m/z [ppm]	Range	Sequence	
419/A1	FETUA_HUMAN	3	554.2599	3	0.89	107 - 120	K.EHAVEGDCDFQLLK.L	CAM: 8
			694.3463	3	0.51	318 - 337	R.HTFMGVVSLGSPSGEVSHPR.K	
			860.4410	4	0.88	188 - 211	R.AQLVPLPPSTYVEFTVSGTDCVAK.E	CAM: 21
428/A2	FETUA_HUMAN	3	407.2295	2	1.48	125 - 131	K.FSVVYAK.C	
			409.5649	3	1.79	121 - 131	K.LDGKFSVVYAK.C	
			554.2592	3	-0.30	107 - 120	K.EHAVEGDCDFQLLK.L	CAM: 8
439/A3	FETUA_HUMAN	7	407.2299	2	2.46	125 - 131	K.FSVVYAK.C	
			521.0115	4	0.37	318 - 337	R.HTFMGVVSLGSPSGEVSHPR.K	
			554.2599	3	0.96	107 - 120	K.EHAVEGDCDFQLLK.L	CAM: 8
			613.8432	2	0.96	121 - 131	K.LDGKFSVVYAK.C	
			677.3390	3	0.64	104 - 120	R.QLKEHAVEGDCDFQLLK.L	CAM: 11
			860.4418	3	1.79	188 - 211	R.AQLVPLPPSTYVEFTVSGTDCVAK.E	CAM: 21
448/A4	FETUA_HUMAN	5	1121.8573	3	2.93	29 - 57	R.QPNCDDPETEEAALVAIDYINQNLPWGYK.H	CAM: 4
			407.2282	2	-1.71	125 - 131	K.FSVVYAK.C	
			521.0111	4	-0.34	318 - 337	R.HTFMGVVSLGSPSGEVSHPR.K	
			554.2593	3	-0.12	107 - 120	K.EHAVEGDCDFQLLK.L	CAM: 8
			613.8428	2	0.30	121 - 131	K.LDGKFSVVYAK.C	
444/A5	FETUA_HUMAN	5	677.3382	3	-0.54	104 - 120	R.QLKEHAVEGDCDFQLLK.L	CAM: 11
			407.2292	2	0.75	125 - 131	K.FSVVYAK.C	
			409.5634	3	-1.87	121 - 131	K.LDGKFSVVYAK.C	
			508.2552	4	-1.07	104 - 120	R.QLKEHAVEGDCDFQLLK.L	CAM: 11
			521.0101	4	-2.26	318 - 337	R.HTFMGVVSLGSPSGEVSHPR.K	
469/A6	FETUA_HUMAN	2	554.2589	3	-0.84	107 - 120	K.EHAVEGDCDFQLLK.L	CAM: 8
			407.2290	2	0.25	125 - 131	K.FSVVYAK.C	
461/A7	FETUA_HUMAN	5	554.2594	3	0.06	107 - 120	K.EHAVEGDCDFQLLK.L	CAM: 8
			407.2289	2	0.01	125 - 131	K.FSVVYAK.C	
			521.0106	4	-1.32	318 - 337	R.HTFMGVVSLGSPSGEVSHPR.K	
			554.2587	3	-1.20	107 - 120	K.EHAVEGDCDFQLLK.L	CAM: 8
			860.4416	3	1.62	188 - 211	R.AQLVPLPPSTYVEFTVSGTDCVAK.E	CAM: 21
			920.2119	4	2.78	68 - 99	K.VWPQQPSGELFEIIDLTTCHVLDPTPVAR.C	CAM: 22

Supplementary Tables 3

Supplementary Table 3. Overview of main Fetuin A tryptic-peptides identified by nanoESI-Qq-TOF MS/MS.

Measured. (Meas.). Peptide charge state (z). Carbamidomethyl Cys (CAM). Phosphorylation (Phospho). Hexose (Hex). N-acetylhexosamine (HexNAc). Sialic acid (NeuAc). Range, numbering according to the pro-protein sequence.

Accession	Chain	m/z meas.	z	? m/z [ppm]	Sequence	Modifications	Glycosylation type	Sialic acid	Range	Label-free MS	Label-free MS (Anova p-value)
FETUA_HUMAN	A	1107.041	4	4.00	APHGPGLIYRQPNCDPETEEAALVAIDYINQNLPGWK.H	CAM: 14			20 - 57		
FETUA_HUMAN	A	1065.0150	4	0.79	P.HGPGLIYRQPNCDPETEEAALVAIDYINQNLPGWK.H	CAM: 12			21 - 57		
FETUA_HUMAN	A	1121.8587	3	4.17	R.QPNCDPETEEAALVAIDYINQNLPGWK.H	CAM: 4			29 - 57		
FETUA_HUMAN	A	1136.0470	4	2.09	R.QPNCDPETEEAALVAIDYINQNLPGWKHTLNQIDEVK.V	CAM: 4			29 - 67		
FETUA_HUMAN	A	1317.6619	2	2.97	D.PETEEAALVAIDYINQNLPGWK.H				35 - 57		
FETUA_HUMAN	A	971.8927	5	2.02	K.HTLNQIDEVKVWPQPSGELFEIETDLETTCHVLDPTPVAR.C	CAM: 32			58 - 99		
FETUA_HUMAN	A	1098.3058	4	2.35	N.QIDEVKVWPQPSGELFEIETDLETTCHVLDPTPVAR.C	CAM: 28			62 - 99		
FETUA_HUMAN	A	508.2569	4	3.86	R.QLKEHAVEGDCDFQLK.L	CAM: 11			104 - 120	X	ns
FETUA_HUMAN	A	554.2603	3	1.70	K.EHAVEGDCDFQLK.L	CAM: 8			107 - 120	X	ns
FETUA_HUMAN	A	519.2541	4	1.81	K.EHAVEGDCDFQLKLDGK.F	CAM: 8			107 - 124		
FETUA_HUMAN	A	407.2298	2	1.59	K.FSVVYAK.C				125 - 131	X	ns
FETUA_HUMAN	A	1326.2169	3	4.29	K.VCQDCPLAPLNDR.V	CAM: 2, 5; Hex(5)HexNAc(4)NeuAc(2): 14	N-linked glycan	2	145 - 159	X	ns
FETUA_HUMAN	A	1544.9553	3	1.03	K.VCQDCPLAPLNDR.V	CAM: 2, 5; Hex(6)HexNAc(5)NeuAc(3): 14	N-linked glycan	3	145 - 159	X	ns
FETUA_HUMAN	A	1183.0825	2	2.00	K.AALAAFNQNNNGSNFQLEIEIS.R				166 - 187		
FETUA_HUMAN	A	1523.9822	3		K.AALAAFNQNNNGSNFQLEIEIS.R	Hex(5)HexNAc(4)NeuAc(2): 11	N-linked glycan	2	166 - 187	X	ns
FETUA_HUMAN	A	1143.2366	4	0.30	K.AALAAFNQNNNGSNFQLEIEIS.R	Hex(5)HexNAc(4)NeuAc(2): 11	N-linked glycan	2	166 - 187	X	ns
FETUA_HUMAN	A	1742.7277	3		K.AALAAFNQNNNGSNFQLEIEIS.R	Hex(6)HexNAc(5)NeuAc(3): 11	N-linked glycan	3	166 - 187		
FETUA_HUMAN	A	1290.1579	2	0.94	R.AQLVPLPPSTYVEFTVSGTDCVAK.E	CAM: 21			188 - 211	X	ns
FETUA_HUMAN	A	1093.8886	3	1.83	R.AQLVPLPPSTYVEFTVSGTDCVAK.E	CAM: 21			188 - 218		
FETUA_HUMAN	A	424.2214	2	0.94	K.CNLLAEK.Q	CAM: 1			219 - 225	X	ns
FETUA_HUMAN	A	401.6823	2	1.99	K.QYGFCK.A	CAM: 5			226 - 231	X	p<0.05
FETUA_HUMAN	A	1741.847	5	2.91	LGGAEVAVTCTVFQTPVTSQPQPEGANEAVPTPVDPDAPPSPLGAPGLPPAGSPDASHVLLAAPPQHLHR	CAM: 10; Hex(1)HexNAc(1)NeuAc(1): 19, 33	O-linked glycan	2	238 - 311	X	ns
FETUA_HUMAN	A	1800.0704	5	4.71	LGGAEVAVTCTVFQTPVTSQPQPEGANEAVPTPVDPDAPPSPLGAPGLPPAGSPDASHVLLAAPPQHLHR	CAM: 10; Hex(1)HexNAc(1)NeuAc(1): 19 or 33 ; Hex(1)HexNAc(1)NeuAc(2): 19 or 33	O-linked glycan	3	238 - 311	X	p<0.01
FETUA_HUMAN	A	1858.2795	5	-1.30	LGGAEVAVTCTVFQTPVTSQPQPEGANEAVPTPVDPDAPPSPLGAPGLPPAGSPDASHVLLAAPPQHLHR	CAM: 10; Hex(1)HexNAc(1)NeuAc(2): 19, 33	O-linked glycan	4	238 - 311	X	p<0.001
FETUA_HUMAN	A	724.3871	5	2.43	D.PDAPPSPLGAPGLPPAGSPDASHVLLAAPPQHLHR.A				275 - 311		
FETUA_HUMAN	A	387.6989	2	0.09	R.AHYDLR.H				312 - 317	X	ns
FETUA_HUMAN	A	568.0879	5	5.50	R.AHYDLRHTFMGVVSLGSPSGEVSHPR.K				312 - 337		
FETUA_HUMAN	A	642.3136	3	2.16	R.HTFMGVVSLGSPSGEVSHPR.R				318 - 336		
FETUA_HUMAN	A	694.3468	3	1.16	R.HTFMGVVSLGSPSGEVSHPR.K				318 - 337	X	ns
FETUA_HUMAN	A	541.0047	4	3.36	R.HTFMGVVSLGSPSGEVSHPR.K	Phospho: 13			318 - 337	X	ns
FETUA_HUMAN	A	721.0030	3	2.16	R.HTFMGVVSLGSPSGEVSHPR.K	Phospho: 13			318 - 337	X	ns
FETUA_HUMAN	A	553.0377	4	5.00	R.HTFMGVVSLGSPSGEVSHPR.T				318 - 338		
FETUA_HUMAN	B	1008.5432	2	4.76	R.TVVQPSVGAAGPVVPPCPGR.I	CAM: 18			341 - 361	X	ns
FETUA_HUMAN	B	1336.6567	2	3.40	R.TVVQPSVGAAGPVVPPCPGR.I	CAM: 18; Hex(1)HexNAc(1)NeuAc(1): 1	O-linked glycan	1	341 - 361	X	ns
FETUA_HUMAN	B	891.4380	3	0.90	R.TVVQPSVGAAGPVVPPCPGR.I	CAM: 18; Hex(1)HexNAc(1)NeuAc(1): 1	O-linked glycan	1	341 - 361	X	ns
FETUA_HUMAN	B	988.4692	3	0.14	R.TVVQPSVGAAGPVVPPCPGR.I	CAM: 18; Hex(1)HexNAc(1)NeuAc(2): 1	O-linked glycan	2	341 - 361	X	p<0.01
FETUA_HUMAN	B	958.0155	2	0.94	T.VVQPSVGAAGPVVPPCPGR.I	CAM: 17			342 - 361		
FETUA_HUMAN	B	908.4811	2	0.81	V.VQPSVGAAGPVVPPCPGR.I	CAM: 16			343 - 361		

Supplementary Figure Legends**Supplementary Figure 1. VO56.07A study design.**

Schematic design of the pollen chamber trial. Following screening and randomization, 82 grass pollen allergic patients were treated sublingually once daily with either a placebo (n=?) or a grass pollen (n=?) tablet for 4 months. Grass pollen challenges were performed before treatment (at inclusion), at day 7, and at months 1, 2 and 4 during AIT, with an evaluation of ARTSS during the 4 hours post-challenge. The search for predictive biomarkers was performed on serum samples collected from patients before treatment initiation.

Supplementary Figure 2. Immunoaffinity enrichment of human FetA.

Visualization of human FetA by SDS-PAGE following Sypro protein staining (A) and western-blot analyses (B). Lanes 1 and 2: 1.5 µg of crude RPMI medium supplemented with 10% human serum, before and after affinity chromatography, respectively; lane 3: 0.3 µg of commercial FetA (BioVendor, Brno, Czech Republic); lane 4: 0.6 µg of FetA obtained by immunoaffinity purification. Lane M: molecular weight markers.

Supplementary Figure 3. Human FetA (B chain) contains sialylated O-linked carbohydrates.

(A) LC-UV analysis of the reduced forms of natural FetA (cf. deconvoluted mass spectrum of the A chain in Figure 2 A. (B-D) MS analysis of FetA B chain. Non-glycosylated (B) and sialylated forms (on O-linked glycans) are observed in the mass spectra (C, D). Yellow square = N-acetylglucosamine; yellow circle = hexose; purple diamond = N-acetylneuraminic acid (sialic acid, Neu5Ac).

Supplementary Figure 4. Confirmation of human FetA desialylation by mass spectrometry.

(A) LC-UV analysis of the reduced forms (using 20 mM DTT) of natural AsialoFetA obtained after treatment of purified native (i.e., sialylated) FetA with neuraminidase. MS analyses of A (B) and B chains (C-D) from FetA and/or AsialoFetA. Yellow square = N-acetylglucosamine; yellow circle = hexose; purple diamond = N-acetylneuraminic acid (sialic acid, Neu5Ac).

Supplementary Figure 5. Correlation between OG3 peptide abundance and spot volumes (sp 428 + 439 + 448) in pre-treatment sera of individual patients.

Spearman correlation results obtained at the individual patient level on pre-treatment blood samples from both placebo and active cohorts, relating FetA O-glycopeptide 3 abundance measured by mass spectrometry and FetA spot volumes assessed by 2D-DiGE.

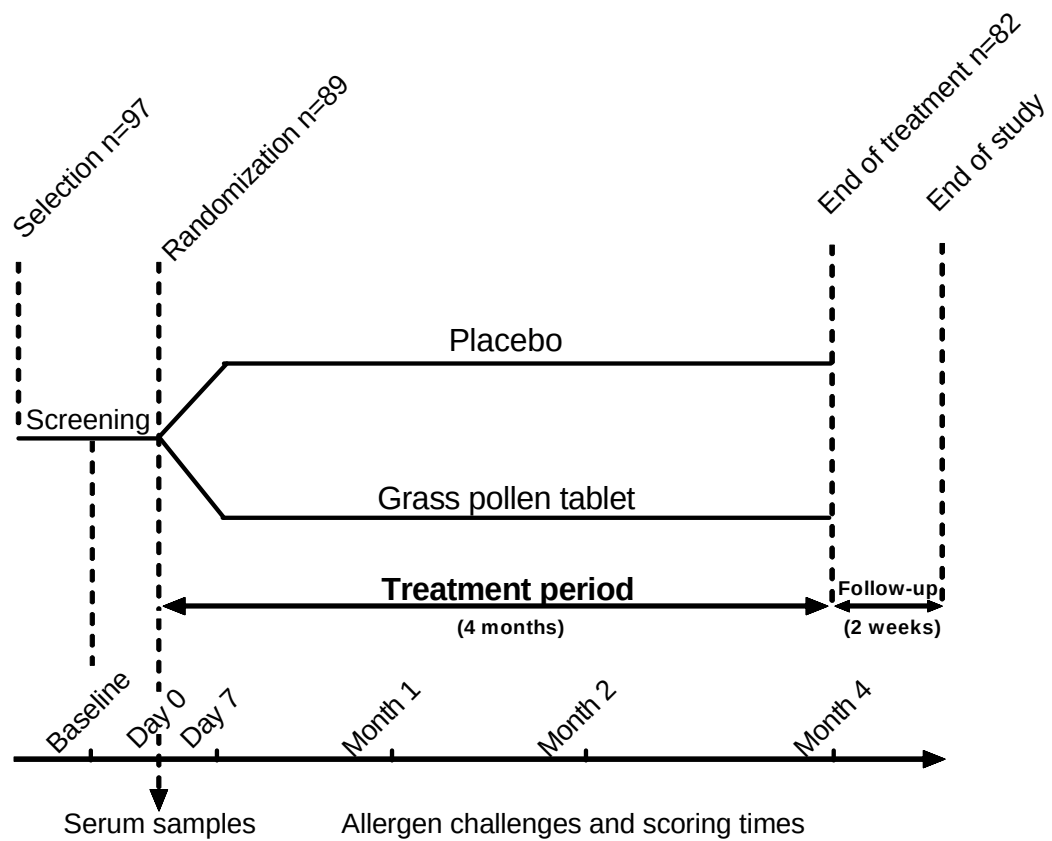
Supplementary Figure 6. Timothy grass pollen sensitization model with concomitant FetA gene silencing.

(A) Naive mice were sensitized intraperitoneally with a timothy grass pollen extract adsorbed on alum. At days 7 and 8, mice received an intranasal administration of a timothy grass pollen extract. To silence FetA expression, mice received intravenous injections of either specific vivo-morpholinos or controls at days -1, 2, 5 and 8. **(B)** Airway hyperresponsiveness by whole body plethysmography was measured in response to increasing concentrations of methacholine, with results expressed as a Penh index as mean values $\pm$ SEM (n = 6 mice per group).

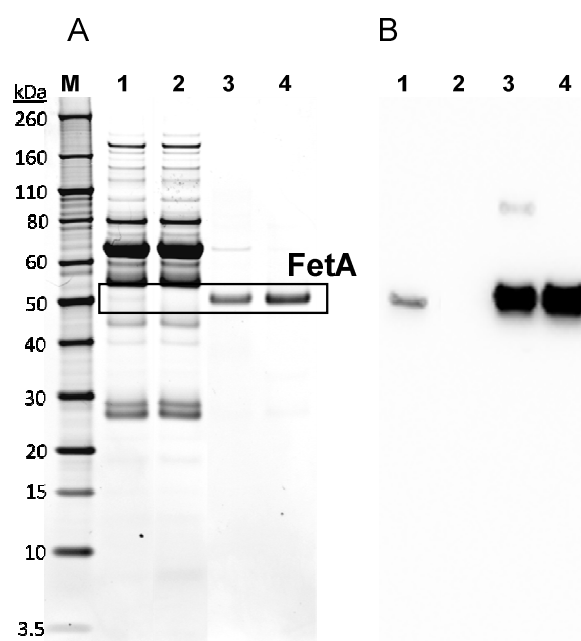
Supplementary Figure 7. Comparison of amino acid sequences of human and murine FetA.

Pairwise alignment of amino-acid sequences of human (UniProtKB entry P02765) and murine (UniprotKB entry P29699) FetA performed with UniProt alignment tool. O-glycosylation sites were confirmed by MS.

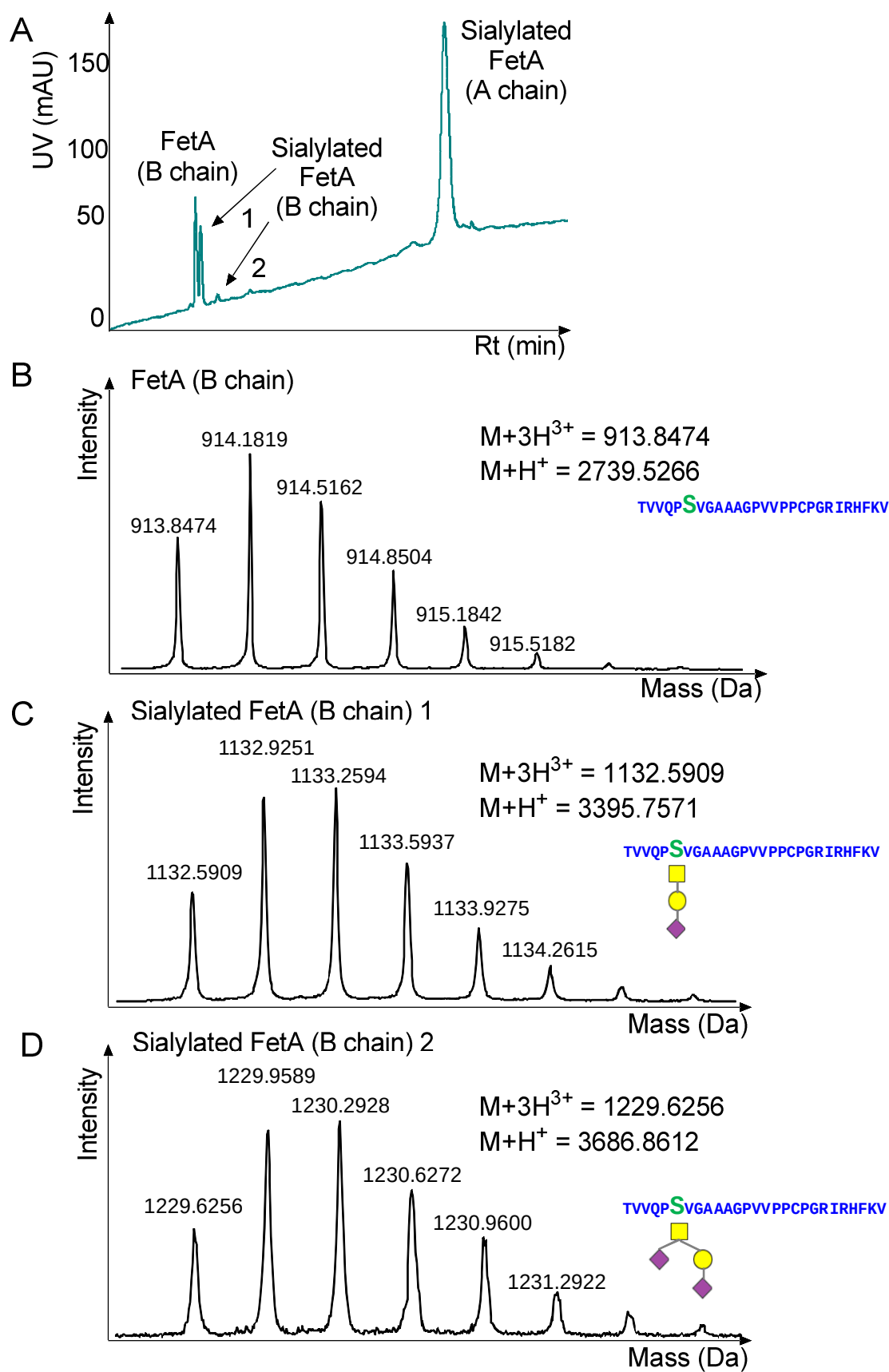
Supplementary Figure 1



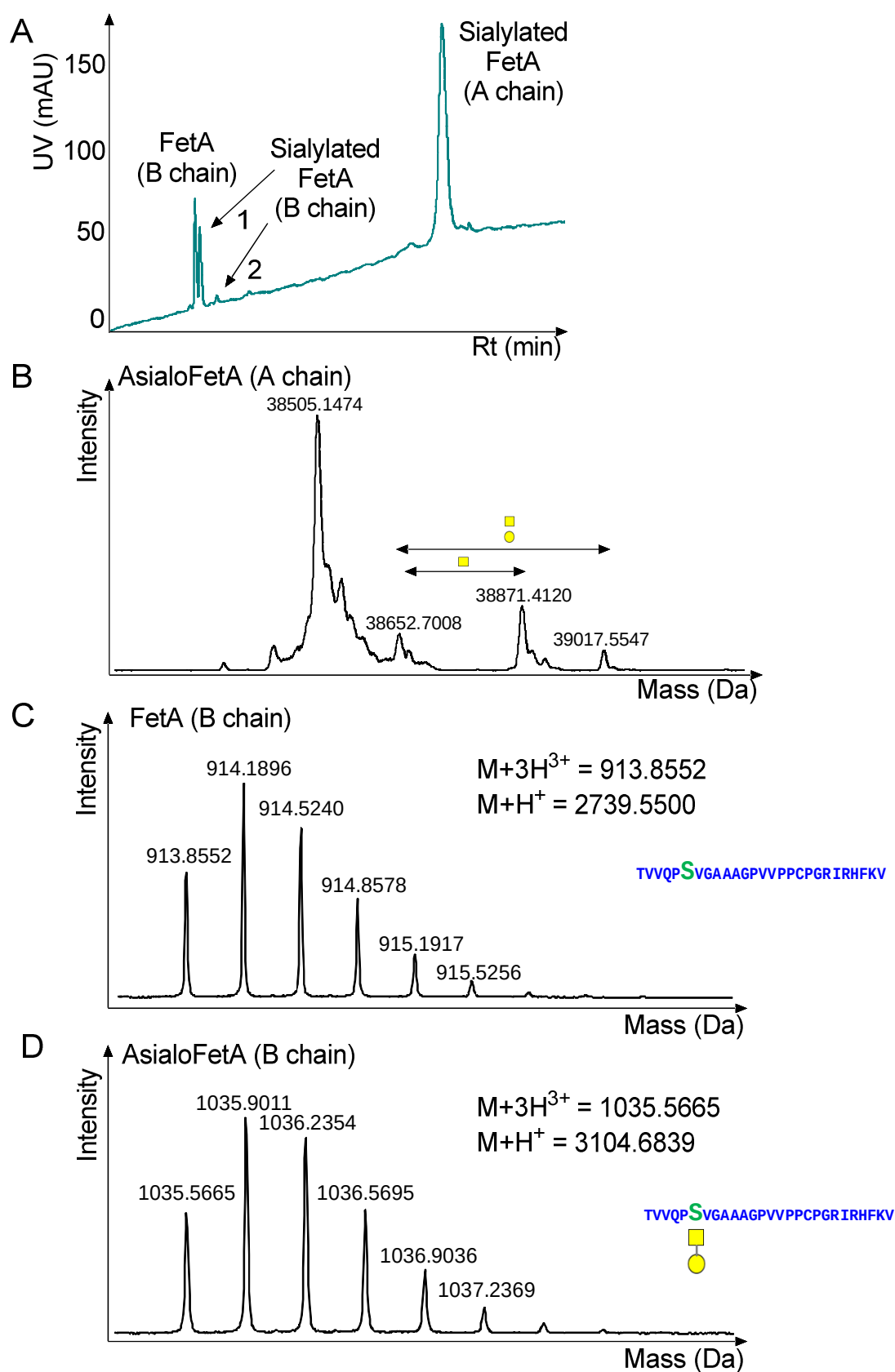
Supplementary Figure 2



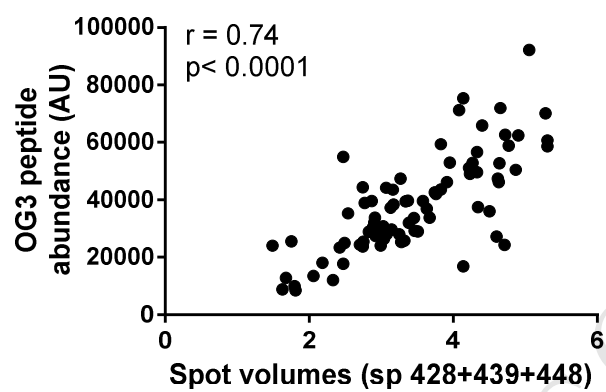
Supplementary Figure 3



Supplementary Figure 4

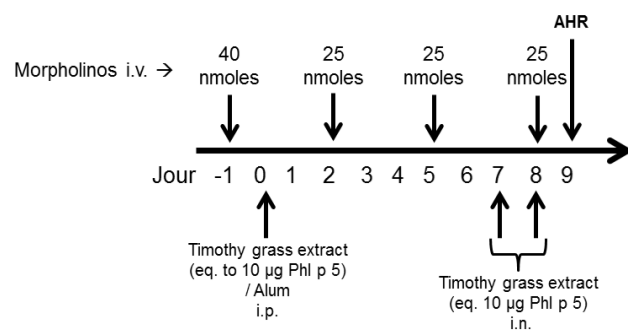


Supplementary Figure 5

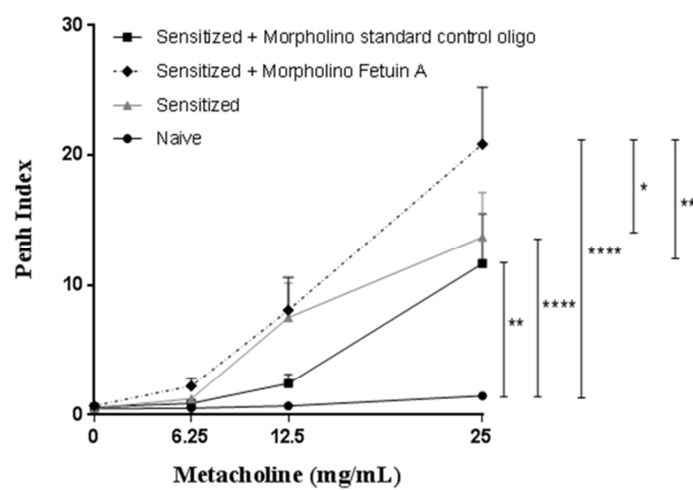


Supplementary Figure 6

A



B



Supplementary Figure 7

