

Combined analytical assays for the characterization of drugs binding to human IgE: Applicability to omalizumab-bearing biosimilar candidates assessment

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ARTICLE INFO

Keywords:

Anti-IgE
Biosimilar
Comparability
Immunoglobulin E
Omalizumab

ABSTRACT

Analytical and functional comparison is key for substantiating the level of convergence (essential sameness) or divergence between versions or variants of a given biological medicine. Accordingly, an overlapping biological activity between products meant to be equal probably reflects a highly similar structure and anticipates a comparable pharmacodynamic behavior. We developed an orthogonal approach to compare the human IgE binding features of different lots and versions of Xolair® (omalizumab), an anti-human IgE monoclonal antibody. The IgE binding affinity and kinetics were measured by surface plasmon resonance. Ability to prevent mast cell activity was assessed in vitro and in vivo in mast cell-based models. The variability of monoclonal antibodies with identical amino acid sequences produced either in Chinese hamster ovarian cells or in human HEK293 cells, was compared. Monoclonal antibodies from the two sources exhibited slightly different human IgE binding and neutralizing features. A known variant exhibiting a three amino acid replacement in the Fab region had lower IgE binding affinity than the original omalizumab. The lower binding affinity translated into reduced IgE neutralizing capacity and, in turn, a difference in the ability to prevent mast cell activation in vitro and in vivo. The proposed set of analytical and functional assays was sensitive enough to detect Fab-linked differences between anti-IgE antibody versions exhibiting an identical amino acid sequence. In addition to add value to the comparative assessment of biosimilar candidates bearing omalizumab, these methods can aid pre-assessments of new anti-IgE agents that aim to improve therapeutic performance.

1. Introduction

Analytical comparative assessment of the biological activities of therapeutic compounds designed to interact with a given target is a cornerstone to predicting their relative pharmacological behaviour. Such assessments are needed to confirm the level of molecular sameness of compounds meant to structurally replicate each other and, to some degree, to predict their pharmacodynamic behavior [1,2]. Comparative

analytical assessment can serve to measure the potency of a novel biological medicine, or more commonly, to gauge the risk of unwanted functional consequences of detected minute molecular differences [3]. Functional analytical comparability is therefore increasingly considered a key to the fine-tuned comparison of versions and/or variants of a given biological medicine [4]. Comparability assessments can also be used to anticipate unwanted impacts of changes in manufacturing processes for both investigational and marketed products [5]. Biological medicines

Abbreviations: ANOVA, analysis of variance; BSA, Bovine serum albumin; CO₂, carbon dioxide; FBS, fetal bovine serum; HC, heavy chain; hIgE, human immunoglobulin E; i.d., intradermal administration; i.v., intravenous administration; IgE, immunoglobulin E; LAD2, Laboratory of Allergic Diseases 2 human mast cells; LC, light chain; LC-MS/MS, liquid chromatography-tandem mass spectrometry; mAb, monoclonal antibody; MC, mast cell; MEM, minimum essential medium; NP, Nitrophenyl; OMA, omalizumab; ON, overnight; PBS, phosphate buffer solution; PCA, passive cutaneous anaphylaxis; PMF, peptide map fingerprint; RS-ATL8, humanized rat basophil leukemia cell line; RSD, relative standard deviation; RT, room temperature; SCF, stem cell factor; SPR, surface plasmon resonance; TOST, two one-sided test; UAB, Autonomous University of Barcelona.

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<https://doi.org/10.1016/j.bioph.2023.115848>

Received 27 June 2023; Received in revised form 25 October 2023; Accepted 5 November 2023

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that are subject to such changes often exhibit unintended physico-chemical differences [6,7] that can only be detected analytically. Indeed, assessment of the functional behavior of pre-change and post-change versions of a compound is a sensitive approach to assessing the relevance of unexpected, or even undetected, minor structural divergence [8,9]. Accordingly, in the context of biosimilar development, where the goal is to demonstrate essential sameness between the original and biosimilar versions of a compound [10,11], the availability of an accurate orthogonal functional analytical approach is a mainstay in substantiating comparability [12,13]. Such an exercise is valuable in predicting the impact of structural differences on the pharmacodynamics, and possibly on the relative efficacy, of biomedicines [14].

The anti-human IgE (hIgE) monoclonal antibody (mAb) omalizumab has been subject to modifications since its original approval for the treatment of severe allergic asthma, chronic idiopathic urticaria, and nasal polyps. A major change in the formulation of Xolair®, the original biological medicine bearing omalizumab, was subjected to an analytical screening to determine if it had any unintended pharmacological or pharmacodynamic effects [15]. In addition, biosimilar candidates that are currently under development need to be subjected to a fine-tuned analytical functional comparison in order to mitigate the risk of overlooking impactful structural differences and predict essentially overlapping clinical features [16,17]. Omalizumab has a straightforward Fab-mediated mechanism of action [18]. Indeed, binding to hIgE is the first biological event leading to the product's therapeutic efficacy [19]. Therefore, comparative assessment of the Fab-mediated hIgE neutralization capacity is a crucial component of the set of studies needed to demonstrate the essential sameness of biosimilars. Regulatory authorities concerned with the science of biosimilarity, such as EMA and MHRA, have fostered a debate on the relevance of functional comparisons to extended comparability assessments [20–22]. In vitro and in vivo tools are available to measure anti-IgE activity; however, technological advances may allow for more precise characterization to ensure a more sensitive comparison of anti-IgE compounds that exhibit minute molecular differences.

To calibrate the discriminating power of methods designed to characterize anti-hIgE biological activity, we analytically scrutinized a set of three analogous anti-hIgE mAbs: the original anti-asthma antibody omalizumab extracted from the medicine Xolair® (OMA_{ref}) produced from Chinese hamster ovary (CHO) cells, used as the comparator; a version meant to have an identical amino acid sequence to OMA_{ref} but produced in a non-CHO eukaryotic expression system (OMA_{ver}), and a variant manufactured in the alternative expression system but bearing a three amino acid replacement (OMA_{var}). Using these three analogous mAbs, we assessed the reliability and sensitivity of a transversal set of analytical and functional comparability studies to detect minute, even clinically irrelevant, differences in hIgE-binding features.

2. Material & methods

2.1. Anti-human IgE monoclonal antibodies

Three anti-hIgE mAbs were scrutinized. OMA_{ref}, the active substance of the biological medicine Xolair® (Novartis), was used as the reference agent. OMA_{ver}, a version of OMA_{ref} expressing the same primary sequence, and OMA_{var}, a variant of OMA_{ref} with a three amino acid replacement, were produced by the company Absolute Antibody Ltd. in HEK293 cells, a cell line originally isolated from the kidney of a human embryo [23]. The alternative amino acid sequence of OMA_{var} was obtained from the original Xolair® patent. To prevent analytical differences driven by the production medium or impurities, the three mAbs were purified and dialyzed into the same buffer at the same concentration. Protein purification and dialysis were performed at *Unique Science and Technology Infrastructures* “NANBIOSIS,” using the Protein Production Platform of the so-called Spanish Biomedical Research Center Network (CIBER) for Bioengineering, Biomaterials &

Nanomedicine, at the Universitat Autònoma de Barcelona SePBioEs scientific technical service (<http://www.nanbiosis.es/portfolio/u1-pr otein-production-platform-ppp/>).

2.2. Primary structure determination

To confirm the primary amino acid sequences of the three mAbs, two sequencing methods were used: de novo sequencing and peptide map fingerprinting.

2.2.1. De novo sequencing

Samples of mAbs were digested with seven combinations of four different enzymes (pepsin, trypsin, chymotrypsin, and Asp N) after processing with disulfide reduction and alkylation. The digestions were analyzed by high-performance liquid chromatography (HPLC) with tandem mass spectrometry (LC-MS/MS) using Thermo Fisher Q Exactive™ and Orbitrap Fusion™ mass spectrometers at the facilities of Rapid Novor Inc. Peptides were characterized from the LC-MS/MS data using de novo peptide sequencing and then assembled into mAb sequences.

2.2.2. Peptide map fingerprinting

Samples containing the mAbs (50 µg at 1 mg/mL) were reduced with 10 mM DTT and 0.1% SDS for 30 min at 60 °C and then alkylated with 55 mM iodoacetamide for 30 min while protected from sunlight. The samples were then precipitated with acetone at – 20 °C for 1 h and reconstituted in 50 µL of 25 mM ammonium bicarbonate. Enzymatic digestion was then performed overnight at 37 °C with trypsin. Analysis was performed on an LC-MS (nanoLC-Velos Orbitrap) at *Centres Científics i Tecnològics de la Universitat de Barcelona* (CCiT-UB). Data were processed with the Thermo Proteome Discoverer 1.4.1.14 software (Thermo Fisher Scientific).

2.3. Anti-IgE/hIgE-binding characterization

Binding affinity for IgE was analyzed and compared by surface plasmon resonance (SPR) using Biacore™ T-200 (GE Healthcare) equipment at CCiT-UB. Anti-IgE antibodies were immobilized on a C1 (Series S) chip by amine coupling (Amine coupling kit, GE Healthcare). The samples containing the mAb under analysis were serially diluted (from 30 nM to 0.47 nM) and rinsed over the chip (association and disassociation time set at 240 s) at 25 °C. Assay cycles were performed in triplicate. The regeneration step was performed with 0.1 M glycine (GE Healthcare) with 0.3% Triton X-100 (Thermo Scientific) at pH 12.0. Kinetic association and dissociation rate constants (k_a and k_d , respectively) and binding constants (K_D) were evaluated using BIA Evaluation software (GE Healthcare Life Science) with a 1:1 binding model. Constants determined for all samples were normalized with an internal standard and reported as relative percentages.

2.4. IgE neutralization bioassays

In vitro and in vivo potency assays were used to compare the impact of the primary mechanism of action of the anti-IgE mAbs on downstream events after ligand binding. The ability of OMA_{ver} and OMA_{var} to prevent mast cell (MC) activity was measured in two MC lines and in an in vivo MC-based model by head-to-head comparison with OMA_{ref}.

2.4.1. Cell-based β -hexosaminidase release assays

LAD2 cells, a human MC line [24], and RS-ATL8 cells, a humanized rat basophilic leukemia cell line [25], were used to conduct β -hexosaminidase release assays to determine the biological activities of OMA_{ver} and OMA_{var} in comparison with that of OMA_{ref}. Both cell lines can be used to assess anti-IgE activity because they both express the human high-affinity IgE receptor (hFcεRI) [26]. LAD2 cells were incubated with anti-IgE mAbs or vehicle (PBS) for 15 min at 37 °C with 5% CO₂ in

SCF-free medium [Stem Pro-34 supplemented with Stem-Pro Nutrient (Gibco), penicillin (100 U/mL), streptomycin (100 µg/mL), and glutamine (2 mM; Sigma)]. The LAD2 cells were then sensitized overnight by adding 100 ng/mL biotinylated hIgE (Abbtotec). Then, the LAD2 cells were washed three times, resuspended in release buffer, and distributed in the wells of a U-bottom 96-well plate (20,000 LAD2 cells/well). RS-ATL8 cells were first transferred to a 96-well culture plate (500,000 RS-ATL8 cells/well) and grown for approximately 36 h at 37 °C with 5% CO₂ in MEM medium supplemented with 10% inactivated FBS, 1% penicillin/streptomycin, 0.5 mg/mL geneticin, and 0.2 mg/mL hygromycin B (Sigma). Anti-IgE mAb or vehicle (PBS) was then added to the RS-ATL8 cell cultures and incubated at 37 °C with 5% CO₂ for 15 min. The RS-ATL8 cells were then sensitized overnight with 500 ng/mL biotinylated hIgE. Following sensitization, both cell lines were washed three times with release buffer. The LAD2 or RS-ATL8 cells were then stimulated with 100 ng/mL or 20 µg/mL streptavidin (Sigma), respectively, for 45 min at 37 °C, after which the cellular activity was stopped by putting the cultures on ice. The cultures were then centrifuged at 250 g for 10 min at 5 °C. Fifty microliters of supernatant was then collected in a lecture plate, and the pelleted cells were lysed by adding 150 µL water and subjecting them to freeze/thaw cycles. Fifty microliters of lysate was then collected in a lecture plate. The amount of extracellular β-hexosaminidase released into the supernatants and lysates by activated MCs was measured (405 nm and 620 nm as reference) as described previously [27] and expressed as a percentage. Antibody activity was expressed as the rate of degranulation inhibition as follows:

Equation 1. Inhibition (%) calculation.

$$\%inhibition = 100 - \frac{(\%_{treatment\ release} - \%_{basal\ release})}{(\%_{total\ release} - \%_{basal\ release})} \times 100$$

%treatment release: Percentage release of the treatment group (anti-IgE mAb).

$$Extravasation(\%) = \frac{LE\ Absorbance_{620} - RE\ Absorbance_{620}}{Mean\ LE\ Abs(+Controls)_{620} - Mean\ RE\ Abs(+Controls)_{620}} \times 100$$

%basal release: Percentage release of the negative controls (groups IgE+/Antigen-, IgE-/Antigen+ and IgE-/Antigen-).

%total release: Percentage release of the positive control (group IgE+/Antigen+).

2.4.2. In vivo passive cutaneous anaphylaxis assays

2.4.2.1. Mice. Passive cutaneous anaphylaxis (PCA) studies were carried out in 8-to-20-week-old humanized FcεRI BALB/c mice (FcεRI^{-/-}hFcεRI⁺) [28]. The average body weight of the mice was 30 g. Male and female mice were combined in each experimental group. All animals were bred at the Animal Facility of UAB from two breeding pairs kindly provided by Dr. Jean-Pierre Kinet at the Beth Israel Deaconess Medical Centre at Harvard University. Animals were housed in groups of 2–6 mice per cage in ventilated racks from Allentown, Inc. (Allentown, NJ, USA), under a 12-h light/dark cycle. Each group of mice was provided bedding material (EcoPure, Datesand), feed pellets (Tecklad, Envigo), and tap water ad libitum. The environmental conditions were controlled and constant during the housing and experiments (temperature: 20 ± 1 °C; humidity: 60 ± 10%). To reduce animal stress, the animals were acclimated for at least 1 week before being used in experiments. All animal procedures were approved by the Ethics Committee for Animal Research at UAB (Spain). These procedures were conducted in accordance with Spanish Directive 53/2013 for the “Protection of animals used in research and for other scientific purposes” of Spanish Legislation and following Directive 2010/63/EU of the

European Parliament and Council.

2.4.2.2. Induction and assessment of passive cutaneous anaphylaxis. Mice were passively sensitized under light isoflurane anesthesia (IsoVet, BBraun) in an anesthetic chamber. Sensitization was carried out by intradermal injection (*i.d.*) injection in the left ear of 20 µL solution containing approximately 300 ng NP-specific hIgE (Chimeric hIgE anti-NP antibody, Bio-Rad). The contralateral ear was injected with 20 µL PBS (Sigma-Aldrich) as a negative control. For the mice used to study the effects of the anti-IgE mAbs, a proportional dose of one of the mAbs was mixed with the hIgE solution injected in the left ear. Before administration, the mixture containing hIgE and anti-IgE mAb was incubated for 20 min at room temperature. Twenty-four hours after the *i.d.* injection, a second dose of anti-IgE mAb equivalent to the first dose was given by intravenous (*i.v.*) injection in the tail vein. One hour later, the mice were challenged with *i.v.* injection through the retro-orbital sinus of a 100 µL PBS solution containing antigen [2 mg/mL NP-BSA (Biosearch Technologies)] and 1% Evans Blue (Sigma-Aldrich). Forty minutes after the antigen injection, the mice were euthanized with CO₂, and both ears were removed and minced. The Evans Blue was then extracted by incubating the minced ear tissues with 700 µL formamide (Sigma-Aldrich) at 55 °C for 2 h. The samples were then centrifuged at 12050 g for 10 min, and 200 µL supernatant was recollected in 96-well lecture plates. The extracted colored solution in the supernatants was assessed at 620 nm using a plate reader spectrophotometer (Spark 10 M, Tecan). The Evans Blue extravasation (%) was expressed as a percentage by subtracting the baseline absorbance in the right ear from the absorbance in the IgE-injected ear (left ear) and dividing by the difference between the mean baseline absorbance and the mean absorbance in the IgE-injected ear of the positive controls (Eq. (2)). The Evans Blue extravasation was then used to calculate the percent inhibition with Eq. (3).

Equation 2. Evans Blue extravasation (%) calculation.

LE = Left ear.

RE = Right ear.

Equation 3. PCA inhibition (%) calculation.

$$Inhibition(\%) = 100 - \frac{(\%_{treatment\ extravasation} - \%_{basal\ extravasation})}{(\%_{total\ extravasation} - \%_{basal\ extravasation})}$$

%treatment extravasation = Extravasation (%) observed in the treatment group.

%total extravasation = Extravasation (%) observed in the positive control group.

%basal extravasation = Basal release (%) of the negative controls.

2.5. Statistical analysis

Statistical significance for the binding assay data was determined using a two-tailed Student's t-test with the software SigmaPlot v14.0 (Systat Software Inc, San Jose, California, USA). Differences among the three anti-hIgE mAbs were evaluated by two-way analysis of variance (ANOVA). The threshold for statistical significance was a p-value < 0.05.

3. Results

3.1. Protein sequences of the anti-hIgE mAbs

OMA_{ref} was sourced from the marketplace, whereas OMA_{ver} and OMA_{var} were produced by a commercial laboratory specifically for our

Table 1

The primary structure of OMA_{ref} and OMA_{ver}. The variable regions of the heavy and light chains are highlighted in black. Both mAbs share an identical amino acid sequence.

HEAVY CHAIN					
EVQLVESGGG	LVQPGGSLRL	SCAVSGYSIT	SGYSWNWIRQ	APGKGLEWVA	SITYDGSTNY
NPSVKGRITI	SRDDSKNTFY	LQMNSLRAED	TAVYYCARGS	HYFGHWHFAV	WGQGTLVTVS
SASTKGPSVF	PLAPSSKSTS	GGTAALGCLV	KDYFPEPVTV	SWNSGALTSG	VHTFPAVLQS
SGLYSLSSVV	TVPSSSLGTQ	TYICNVNHKP	SNTKVDKKVE	PKSCDKTHTC	PPCPAPELLG
GPSVFLFPPK	PKDTLMISRT	PEVTCVVVDV	SHEDPEVKFN	WYVDGVEVHN	AKTKPREEQY
NSTYRVVSVL	TVLHQDWLNG	KEYKCKVSNK	ALPAIEKTI	SKAKGQPREP	QVYTLPPSRE
EMTKNQVSLT	CLVKGFYPSD	IAVEWESNGQ	PENNYKTTTP	VLDSDGSFFL	YSKLTVDKSR
WQQGNVFSCS	VMHEALHNHY	TQKLSLSLSPG	K		
LIGHT CHAIN					
DIQLTQSPSS	LSASVGDRTV	ITCRASQSDV	YDGDSYMNWY	QKPGKAPKIL	LIYAASYLES
GVPSRFSGSG	SGTDFLTITIS	SLQPEDFATY	YCQQSHEDPY	TFGQGTKEVI	KRTVAAPSVF
IFPPSDEQLK	SGTASVCLL	NNFYPREAKV	QWKVDNALQS	GNSQESVTEQ	DSKDYSTYLS
STLTLSKADY	EKHVYACEV	THQGLSSPVT	KSFNRGEC		

experiments. Prior to assessing their Fab-mediated anti-IgE functions, we confirmed the amino acid sequences of the three mAbs. We evaluated the amino acid sequences of OMA_{ref} and OMA_{ver} by de novo sequencing, which is able to identify even isobaric differences in amino acid residues. The de novo sequencing analysis showed that OMA_{ref} exhibited one of the sequences protected in the original Xolair® patent (Table 1) [29]. The de novo sequencing allowed for the identification of 100% of the amino acid residues by at least five peptide scans. As expected, OMA_{ver} shared 100% amino acid sequence identity with OMA_{ref}. Furthermore, both OMA_{ref} and OMA_{ver} presented an N-linked glycosylation site on the heavy chain (HC) constant region at Asn301. No O-linked glycans were detected in either mAb. We also compared the OMA_{var} sequence head-to-head with the OMA_{ref} sequence by peptide map fingerprinting, which did not provide full sequence coverage. The peptide map fingerprinting analysis identified 68% of the OMA_{ref} HC residues and 87% of the OMA_{ref} light chain (LC) residues in OMA_{var}. Despite the limited coverage of OMA_{var} sequencing (68% for the HC and 71% for the LC), the intended three amino acid replacement of the variable region described in the Xolair® patent was identified (Table 2).

3.2. Characterization of mAb-to-hIgE binding

We used SPR analysis to determine if there were differences to measure in the Fab-binding features of the anti-hIgE mAbs depending on the production method (CHO cells versus HEK293 cells) or the presence of the three amino acid replacement (OMA_{var}).

3.2.1. Comparative IgE binding kinetics of the anti-hIgE mAbs

The mAb-to-hIgE binding kinetics of OMA_{ref}, OMA_{ver}, and OMA_{var} are shown in Fig. 1A. The k_a values among the OMA_{ref} samples obtained from five Xolair® lots ranged from a minimum of 0.93 (normalized value equivalent to $2.21 \cdot 10^6$ 1/M·s) to a maximum of 1.06 ($2.52 \cdot 10^6$ 1/M·s). The k_d values of OMA_{ref} ranged from 0.84 ($2.04 \cdot 10^{-3}$ 1/s) to 1.18 ($2.89 \cdot 10^{-3}$ 1/s). Thus, for OMA_{ref}, the relative standard deviation (RSD, or coefficient of variation) of the association constant k_a was 10% and that of the disassociation constant k_d was 13%. For the three lots of OMA_{ver} tested, the RSD was 17% for both k_a and k_d , indicating higher inter-lot variability than OMA_{ref}. The k_a values for two of the OMA_{ver} lots (1 and 3) fell within the reference range of OMA_{ref}, whereas lot 2 exhibited a k_a (1.22 or $2.90 \cdot 10^6$ 1/M·s) that was slightly higher than the

reference range. The k_d values for all three OMA_{ver} lots fell within the reference range. The k_a value for the single batch of OMA_{var} was 0.63 ($1.49 \cdot 10^6$ 1/M·s), which was distinctly below the values for OMA_{ref} and OMA_{ver}. There was almost no variability in the k_a value for OMA_{var} (RSD of 1%). The k_d for OMA_{var} was 1.69 ($4.12 \cdot 10^{-3}$ 1/s) with a variability of 15% RSD.

3.2.2. Comparative IgE binding affinities of the anti-hIgE mAbs

The results for the relative binding affinities were similar to those for the relative binding kinetics. The individual K_D values of all analyzed lots (five lots of OMA_{ref}, three lots of OMA_{ver}, and 1 lot of OMA_{var}) are shown in Fig. 1B. The average K_D of the five OMA_{ref} lots was set to 1.0, and the average K_D of the three OMA_{ver} lots and the K_D of the single OMA_{var} lot were normalized accordingly (Table 3). The individual K_D values of the five OMA_{ref} lots ranged from 0.7 to 1.3 (corresponding to normalized values of $7.52 \cdot 10^{-10}$ M to $1.31 \cdot 10^{-9}$ M) with an RSD of 13%. The K_D values of the three OMA_{ver} lots ranged from 0.6 to 1.3 ($6.36 \cdot 10^{-10}$ M to $1.32 \cdot 10^{-9}$ M) with an RSD of 22%, indicating higher inter-lot variability compared with OMA_{ref}. Although the average K_D was 10% lower for OMA_{ver} than for OMA_{ref}, the TOST assessment did not indicate a significant difference between OMA_{ref} and OMA_{ver}. The single OMA_{var} lot had a K_D of 2.7 ($2.77 \cdot 10^{-9}$ M), representing a 170% increase compared with OMA_{ref}. The TOST assessment indicated that OMA_{var} recognizes hIgE but with a significantly lower binding capacity than OMA_{ref} and OMA_{ver}.

3.3. Biological neutralizing activities of the anti-hIgE mAbs

3.3.1. Comparative cell-based biological activities of the anti-hIgE mAbs

We compared the ability of the three mAbs (OMA_{ref}, OMA_{ver}, and OMA_{var}) to prevent MC activation by neutralizing hIgE in two mammalian cell lines expressing the human high-affinity IgE receptor (hFcεRI). Mast cells from both cell lines (LAD2 and RS-ATL8) release hexosaminidase upon human IgE driven stimulation (Fig. 2). Both activation and OMA_{ref}-mediated inhibition are stronger in LAD2 cells.

3.3.1.1. LAD2 cells. First, we tested the lot-to-lot variability in the inhibitory effects of four separate OMA_{ref} lots on LAD2 cell activation after hIgE sensitization in three independent experiments (Fig. 3). No statistical differences in the inhibitory effects were found among the four

Table 2

Primary structure of the variable region of the heavy chain of OMA_{var}, bearing a three amino acid replacement. Aside from those three amino acids (highlighted in yellow), the remaining sequence of the heavy chain and the full sequence of the light chain are shared with OMA_{ref}.

HEAVY CHAIN					
EVQLVESGGG	LVQPGGSLRL	SCAVSGYSIT	SGYSWNWIRQ	APGKGLEWVA	SITYDGSTNY
ADSVKGRFTI	SRDDSKNTFY	LQMNSLRAED	TAVYYCARGS	HYFGHWHFAV	WGQGTLVTVS
S					

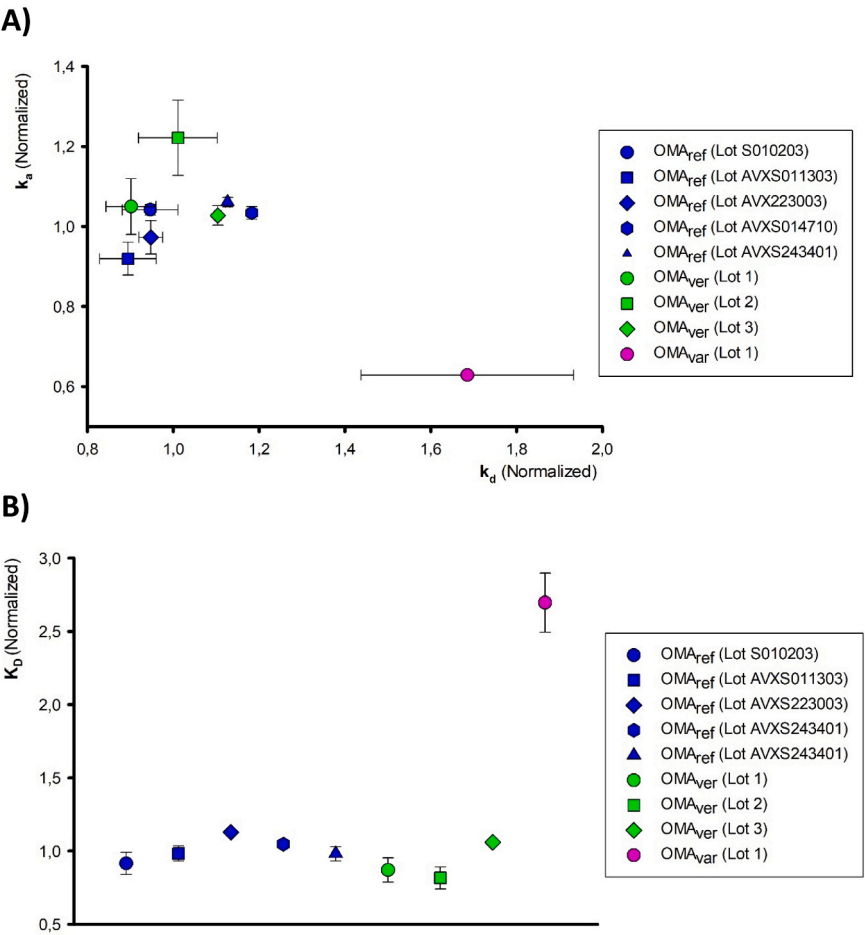


Fig. 1. Representation of the kinetics constants of the three anti-IgE mAbs analyzed by surface plasmon resonance (SPR). **(A)** Graph displaying the association constant (k_a) and dissociation constant (k_d) per analyzed lot. **(B)** Graph showing the affinity constant (K_D) for each analyzed lot. Each lot was assessed four times (4 independent assays), except for the OMA_{ref} AVXS011303 lot which was assessed 10 times. Each point represents the mean $\pm$ standard error of the mean (SEM) of the normalized values.

Table 3
Summary of the binding kinetics of OMA_{ref}, OMA_{ver}, and OMA_{var}. Normalized K_D values and ranges from the kinetics analysis of different batches of the three anti-IgE compounds are provided. The normalized data are expressed as the relative potency of a sample compared with the reference standard (OMA_{ref}). TOST analyses were performed using OMA_{ref} as reference. K_D (affinity constant), SD (standard deviation), TOST (Equivalence Testing – Two one-sided tests).

Normalized K_D	Mean	Range	SD	TOST Result
OMA _{ref}	1.0	0.7–1.3	0.1	Not applicable
OMA _{ver}	0.9	0.6–1.3	0.2	Equivalent
OMA _{var}	2.7	2.4–3.2	0.4	No equivalent

lots. We then compared the inhibitory effects of OMA_{ref} and OMA_{ver} on LAD2 cells (Fig. 4A). The log-transformed dose-response curve of OMA_{ref} exhibited a sigmoid profile. The concentration is expressed on the X-axis in Fig. 4 as the ratio between the amount of OMA_{ref} or OMA_{ver} applied and the quantity of hIgE administered in the sensitization step. At low concentrations, OMA_{ref} exhibited a baseline inhibitory capacity below 10%. At a 30:1 concentration, the inhibition increased rapidly with a highly reactive response to minor concentration changes, reaching a plateau (100% inhibition) at a concentration of 150:1. OMA_{ver} showed a similar inhibitory profile, resulting in an EC50 value close to that of OMA_{ref} (OMA_{ref} EC50 = 111:1; OMA_{ver} EC50 = 102:1). The dose-response curves of OMA_{ref} and OMA_{ver} essentially overlapped with no statistically significant differences between them when tested by ANOVA. OMA_{var} exhibited a similarly shaped sigmoidal dose-response

curve and had the same inhibitory capacity as OMA_{ref} at the lowest and highest concentrations (Fig. 4B). However, OMA_{var} had lower inhibitory potency than OMA_{ref} at the intermediate concentrations, resulting in a different EC50 value (OMA_{ref} EC50 = 24:1; OMA_{var} EC50 = 91:1). Although both mAbs reached full inhibition, OMA_{ref} reached maximum inhibitory capacity (almost 100%) at a 100:1 concentration, whereas OMA_{var} had to be applied at a 300:1 concentration to have the same inhibitory effect (ANOVA p-value = 0.004).

3.3.1.2. RS-ATL8 cells. Preliminary experiments revealed that RS-ATL8 cells present higher intrinsic variability than LAD2 cells. We did not assess the inter-lot variability of OMA_{ref} in RS-ATL8 cells. As in the assays with LAD2 cells, OMA_{ref} and OMA_{ver} had similar sigmoid-shaped dose-response curves when applied to RS-ATL8 cells (Fig. 5A). Neither mAb caused any basal inhibition at the lowest concentrations. The inhibitory activity of both mAbs started at a 3:1 concentration and increased exponentially until reaching full inhibition at a 100:1 concentration. OMA_{ref} and OMA_{ver} presented similar EC50 values (OMA_{ref} EC50 = 25:1; OMA_{ver} EC50 = 16:1). Although ANOVA did not indicate statistical differences in inhibitory capacity between OMA_{ref} and OMA_{ver}, OMA_{ver} tended to have a slightly stronger inhibitory effect at every concentration tested. When the inhibitory effect of OMA_{ref} in RSATL8 cells was compared with that of OMA_{var}, OMA_{ref} presented baseline inhibitory activity at a 30:1 concentration, whereas the lowest concentration at which OMA_{var} had an inhibitory effect was 100:1, a concentration that resulted in 100% inhibition by OMA_{ref} (Fig. 5B).

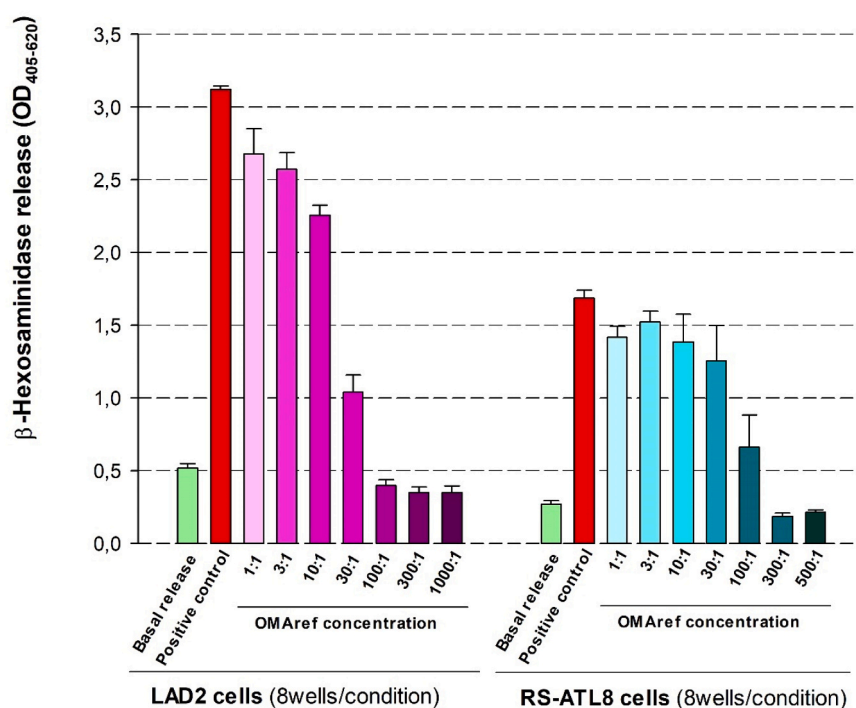


Fig. 2. LAD2 and RS-ATL8 mast cell populations activity. β -hexosaminidase release is expressed as optical density (OD) values at 405 nm, and reference at 620 nm. The graph displays the observed supernatant absorbances of two sets of experiments, one per cell line, subjected to a range of OMA_{ref} concentrations. The concentration of OMA_{ref} is expressed as the ratio between OMA_{ref} and human IgE. Each condition in both experiments was assessed in 8 different assays. The OD values are expressed as the mean $\pm$ standard error of the mean (SEM).

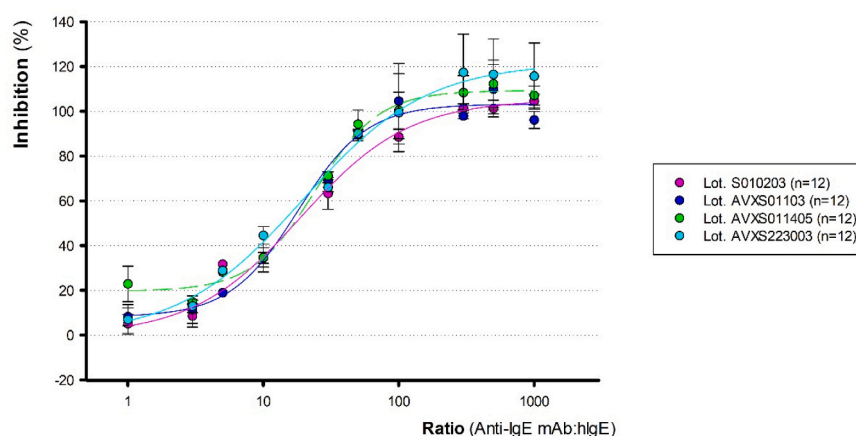


Fig. 3. Lot-to-lot variation in the inhibitory effect of OMA_{ref} on LAD2 cell activation after hlgE sensitization. Each point represents the mean of three independent experiments performed in quadruplicate. Error bars represent the standard error of the mean. Curves were fitted using SigmaPlot v14.0.

OMA_{var} had to be applied at a 500:1 concentration to reach its full inhibitory effect. Overall, the dose-response curves of OMA_{var} and OMA_{ref} were significantly different (ANOVA $p = 0.018$), which translated into a lower EC₅₀ value for OMA_{ref} (48:1) than for OMA_{var} (185:1).

3.3.2. Comparative in vivo biological activities of the anti-IgE mAbs

We next assessed the transferability of the in vitro outcomes to an in vivo setting using a PCA murine model. The PCA mouse model is an MC-dependent model that allows for the assessment of anti-IgE antibody activity in vivo. We simultaneously assessed the in vivo activities of OMA_{ref}, OMA_{ver}, and OMA_{var} at five doses. The inhibitory effects are displayed in Fig. 6 (see representative pictures of the mice's ear swelling,

i.e. Evans Blue extravasation, in [Supplementary Material](#)). All negative controls presented a minimal baseline Evans Blue extravasation equal to or below 6% relative to the positive control (normalized as 100%). OMA_{ref} exhibited a dose-response correlation in vivo. At the lowest dose of 0.3:1 (0.3 μ g OMA_{ref} per 1 μ g IgE), OMA_{ref} inhibited local inflammation by 17%. The inhibitory effect of OMA_{ref} increased until it reached 80% at the highest dose (30:1). OMA_{ver} exhibited similar effects, with no statistically significant differences in inhibition across the five concentrations compared to OMA_{ref}. At the lowest and highest doses, OMA_{ver} inhibited MC-dependent ear edema by 20% and 97%, respectively. OMA_{var} also exhibited dose-response behavior in vivo; however, its inhibitory effect was weaker across the five doses compared

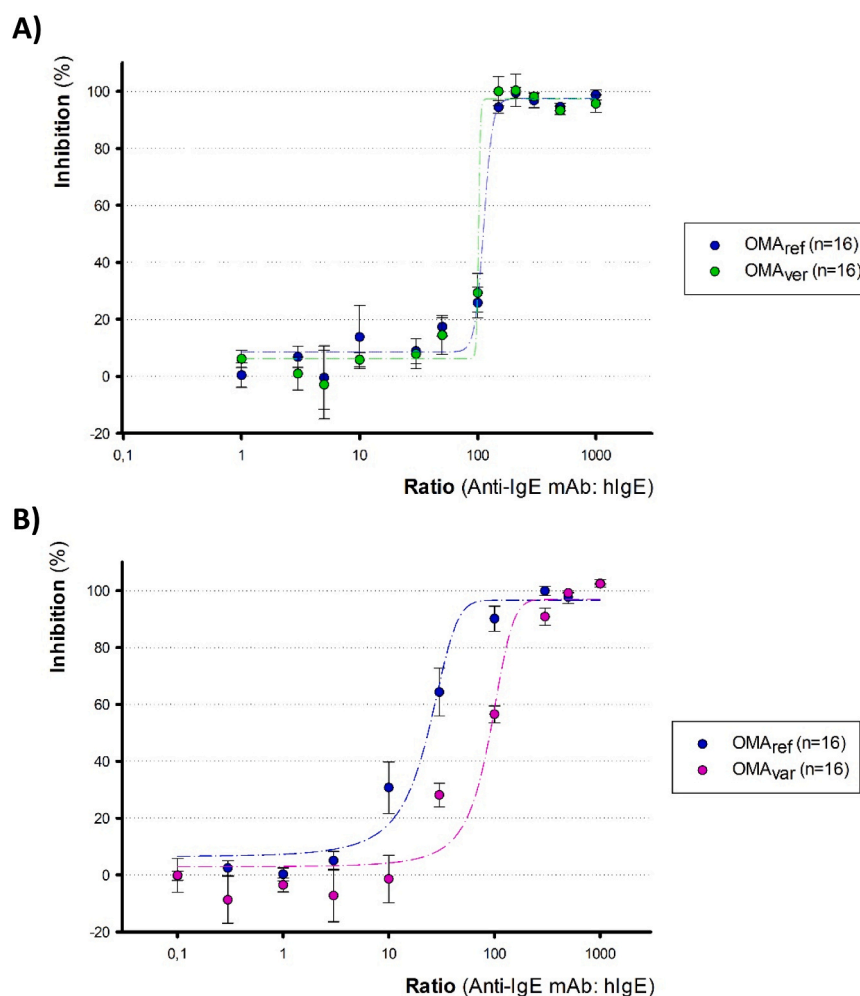


Fig. 4. Head-to-head comparison of the dose-dependent inhibitory effects of the mAbs on LAD2 cell degranulation. Cells were sensitized with 100 ng/mL hIgE, treated with different concentrations of anti-IgE mAbs, and then stimulated with 100 ng/mL antigen. Concentrations of mAbs are expressed as the ratio of the quantities of mAb and IgE administered. **(a)** OMA_{ref} compared with OMA_{ver}. **(b)** OMA_{ref} compared with OMA_{var}. Each point represents the mean of four independent experiments performed in quadruplicate. Error bars represent the standard error of the mean. Curves were fitted using SigmaPlot v14.0.

with those of OMA_{ref} and OMA_{ver} (ANOVA *p*-value < 0.05 for both comparisons), reaching a maximum inhibitory effect of only 41%.

4. Discussion

Our proposed comparative transversal analytical assessment of the hIgE-binding features was suitable to uncover the impact of slight structural differences. Omalizumab samples produced commercially in CHO cells (OMA_{ref}) or alternative anti-IgE mAb produced in HEK293 cells with or without a three amino acid change in primary structure (OMA_{var} or OMA_{ver}, respectively) were studied. The orthogonal analytic approach, including SPR and MC-based *in vitro* and *in vivo* assays, provided sufficient sensitivity to functionally discriminate between anti-IgE compounds that share an identical amino acid sequence.

Different batches of omalizumab obtained from the marketed product Xolair® exhibited minute differences in their binding properties to hIgE. Although small differences uncovered by SPR, may be attributable to the equipment variability, batch-to-batch molecular variation is known to be intrinsic to biotechnology-derived medicines, including complex glycoproteins such as mAbs [30]. For instance, post-translational quantitative and/or qualitative differences in glycan expression can impact the fine-tuned pharmacological behavior of biomedicines [31,32]. Such variability may be inherent to any original biological compound, but it is unlikely to have a significant clinical

impact. To our knowledge, no prior study has reported on the clinically acceptable variation of IgE binding kinetics among different lots of commercially produced omalizumab. Our study sets a reference for variability in the Fab function of omalizumab, notwithstanding the fact that for a more accurate determination of the lot-to-lot variation, at least 10 lots should be scrutinized.

Our comparative studies of the functional properties of OMA_{ref} and OMA_{ver} suggest that Fc-linked glycans may impact the affinity and/or kinetics of interaction between omalizumab and IgE. Indeed, although OMA_{ref} and OMA_{ver} share an identical amino acid sequence and may therefore be considered essentially the same protein, the different expression systems used for their production (CHO or HEK293 cells) might have resulted in different post-translational modifications, and possibly different spatial conformations affecting Fab structure [33]. The functional differences between OMA_{ref} and OMA_{ver} did not reach statistical significance; however, the consistent trend of higher potency for OMA_{ver} irrespective of the assay used to assess the anti-IgE effect (SPR or MC-based *in vitro* or *in vivo* assays) supports the hypothesis that two anti-IgE mAbs with an identical amino acid sequence can exhibit slight functional differences. It is unlikely that excipients or diluents explain the trend, because they were the same in all the analyzed agents. Post-translational modifications or protein folding features might also explain such slight functional divergence [34], but this should be uncovered in a different study. Noticeably, LAD2 mast cells seem a more

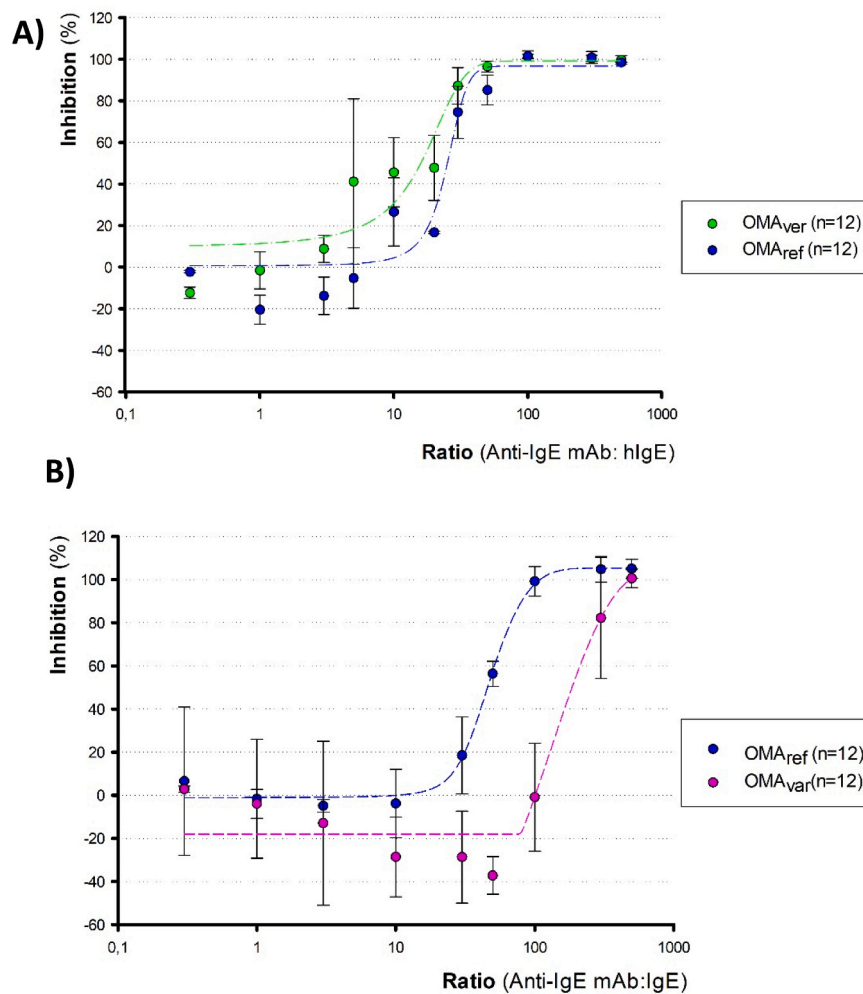


Fig. 5. Head-to-head comparison of the dose-dependent inhibitory effects of the mAbs on RS-ATL8 cell degranulation. Cells were sensitized with 500 ng/mL hIgE, treated with different concentrations of anti-IgE mAbs, and then stimulated with 20 μ g/mL antigen. Concentrations of mAbs are expressed as the ratio of the quantities of mAb and IgE administered. (a) OMA_{ref} compared with OMA_{ver}. (b) OMA_{ref} compared with OMA_{var}. Each point represents the mean of three independent experiments performed in quadruplicate. Error bars represent the standard error of the mean. Curves were fitted using SigmaPlot v14.0.

sensitive in vitro setting than RS-ATL8 mast cells for the purpose of comparing the potency of anti-IgE agents, since it exhibits both a stronger reactivity to IgE-driven stimulation and a higher inhibition in the presence of IgE neutralizing agents. Interestingly, despite the fact that in vivo studies are less prone to reveal minor functional differences than in vitro studies, the MC-dependent PCA model in transgenic mice expressing the human IgE receptor revealed the same trend observed in the cell-based studies, namely, a higher inhibitory capacity and more erratic functioning of OMA_{ver} (whose production, contrary to that of OMA_{ref}, is not good manufacturing practice (GMP) compliant). All in all, the head-to-head comparison of the activities of OMA_{ref} and OMA_{ver} suggests that post-translational modifications, or differences in spatial conformation, may play a role in the ability of omalizumab to neutralize hIgE. A detailed co-assessment of the glycopattern and the anti-IgE mechanism of action will be key to substantiating the relevance of the glycopattern to the biological activity of omalizumab.

The combined use of SPR, in vitro cell-based assays, and the in vivo PCA model proved to be a sensitive approach to characterize the binding properties of anti-IgE proteins meant either to replicate each other or to differ in potency. Such differences, or similarities, may anticipate differences or similarities in relative clinical benefit. To confirm the suitability of our approach, we evaluated an anti-IgE mAb with a known molecular difference compared with the reference mAb. OMA_{var} is an anti-hIgE mAb whose primary sequence is described in the Xolair®

patent to also exert anti-IgE activity. It exhibits a three amino acid replacement in relation to the marketed mAb in the variable region of the HC. In addition, OMA_{var} may also have a divergent glycopattern compared with OMA_{ref} owing to its production in HEK293 cells rather than CHO cells. Although OMA_{var} has the same three amino acid differences compared with both OMA_{ref} and OMA_{ver}, but its glycopattern may be more similar to that of OMA_{ver} because of the shared expression system and production process. Head-to-head comparison of OMA_{var} and OMA_{ver} showed that the small difference in amino acid sequence conclusively translated into a worsening of the mAb binding kinetics and hIgE recognition. Interestingly, the outcomes of the three methods used to assess the Fab-related functions of the mAbs were correlated. As the magnitude of molecular differences became greater, going from minute batch-to-batch differences to likely wider differences and finally to primary structural differences, the magnitude of the differences in hIgE binding and neutralization increased accordingly. These functional differences were detected with all three experimental approaches, and the magnitudes of the differences were correlated across experimental systems. Contrary to the common belief that in vivo approaches have low sensitivity to detect small molecular differences, the transgenic mouse model was able to uncover the differences in biological activity. An orthogonal comparison of anti-hIgE compounds can confidently anticipate the in vivo behavior of the compounds because the binding to the target is known to explain the therapeutic benefit. It is worth mentioning

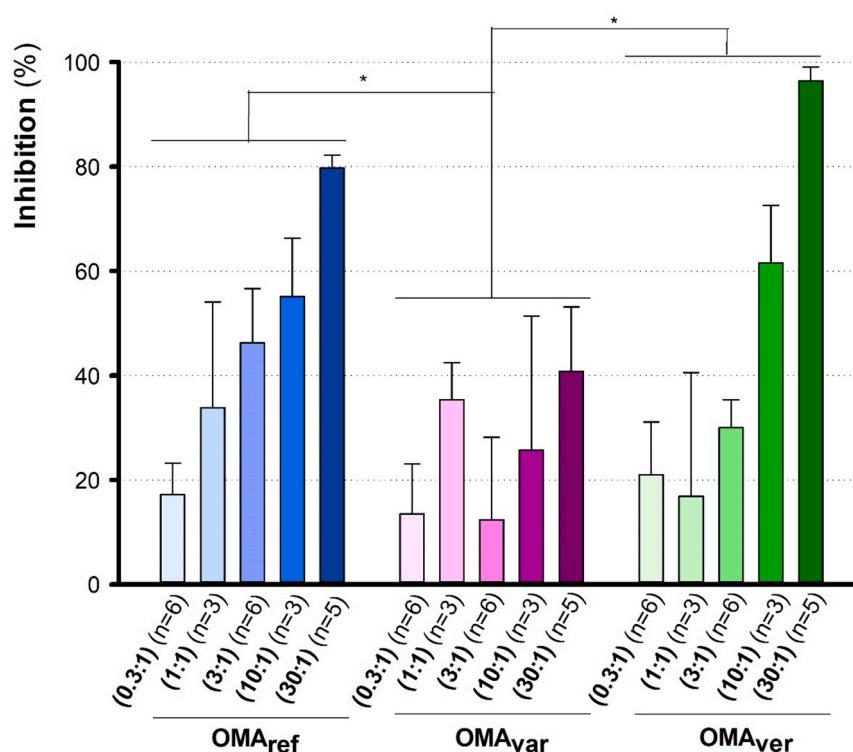


Fig. 6. Comparison of the inhibitory effects of five different concentrations of OMA_{ref}, OMA_{ver}, and OMA_{var} on passive cutaneous anaphylaxis in TG mFcεRI^{−/−}/hFcεRI⁺ BALB/c mice. Concentrations are expressed as the ratio of the amount (μg) of anti-IgE mAb administered and the amount (μg) of IgE dispensed in the sensitization. The inhibition (%) is expressed as the mean ± standard error of the mean. Batches studied: OMA_{ref}, AVXS011405; OMA_{ver}, Lot1; OMA_{var}, Lot2. (*p-value<0.05).

that our proposed anti-IgE functional characterization might be useful in setting international standards for drugs with such a mechanism of action. Indeed, the World Health Organization has recognized a need for global harmonization of the bioactivity of mAbs as new versions of various mAbs are increasingly produced [35].

The objective of our study was to propose a valid and accurate experimental design to uncover the functional relevance of minor molecular alterations in biomedicine. Our three-way comparability approach may apply to different scenarios; for instance, to screen for unwanted functional impacts of changes in manufacturing processes, or to calibrate the difference in binding kinetics and/or affinity of a new product meant to exhibit a higher potency than a similar existing product. Such preliminary analytical functional testing might reduce the failure rate of preclinical or clinical studies. Indeed, innovative anti-IgE molecules such as quilizumab, which did not reach the marketplace because of its limited clinical impact, could benefit from such a pre-clinical functional assessment of their Fab-mediated human IgE neutralization [36]. In particular, fine-tuned functional analytic comparisons under stringent methodological conditions may be of substantial value in the development of biosimilar candidates (i.e., replicas of marketed biological medicines such as Xolair®). A key step in biosimilar development is the non-clinical functional comparison of a biosimilar candidate and its original counterpart. There are currently no marketed biosimilars of Xolair®, but two such biosimilars might be on the way [37]. Our analytic approach provides a fine-tuned functional comparison that anticipates the biosimilarity of different compounds. This can benefit not only products under development but also marketed medicines in need of quality controls to monitor potential drift during their commercial lifecycle.

In conclusion, we proposed and tested a sensitive transverse orthogonal analytic approach to compare the potency of different versions of omalizumab. This approach can be used for new anti-human IgE agents, whether they are full antibodies, Fab fragments, or IgE receptor-

targeted compounds. A standardized combination of the methods used here will provide a more accurate picture of likely pharmacological behavior, and may to some extent anticipate reciprocal efficacy; however, only a more extensive “fit-for-purpose” analytical comparison will be able to predict clinical sameness.

CRediT authorship contribution statement

Adrián Urbano: Experiments execution, Data interpretation, Formal analysis, Manuscript writing, **Judith Plaza:** Experimental collaboration, **César Picado:** Conceptualization, Data interpretation. **Fernando de Mora:** Conceptualization, Data interpretation Writing – original draft preparation, Project administration, Supervision.

Declaration of Competing Interest

Urbano, Adrián: Declaration of Interest: None, Plaza, Judith: Declaration of Interest: None, Picado, César: Declaration of Interest: None, de Mora, Fernando: Declaration of Interest: None.

Acknowledgement

This study was supported by a grant from the Spanish Fondo de Investigación Sanitaria (ref. PI18–01702), managed by the Instituto de Salud Carlos III of the Spanish Ministry of Health, Social Services, and Equality.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2023.115848](https://doi.org/10.1016/j.biopha.2023.115848).

References

- [1] Magnenat L., Palmese A., Fremaux C., Amici F.D., Terlizze M., Rossi M., et al. Demonstration of physicochemical and functional similarity between the proposed biosimilar adalimumab MSB11022 and Humira®, 2017; 0862. Available from: <https://doi.org/10.1080/19420862.2016.1259046>.
- [2] X. Wang, Z. An, W. Luo, N. Xia, Q. Zhao, Molecular and functional analysis of monoclonal antibodies in support of biologics development, *Protein Cell* [Internet] 9 (1) (2018) 74–85 (Available from), (<http://link.springer.com/10.1007/s13238-017-0447-x>).
- [3] A. Ambrogelly, S. Gozo, A. Katiyar, S. Dellatore, R. Bhat, J. Sun, et al., Analytical comparability study of recombinant monoclonal antibody therapeutics, MABs [Internet] 10 (4) (2018) 513–538, <https://doi.org/10.1080/19420862.2018.1438797>.
- [4] H.P. Hofmann, U. Kronthaler, C. Fritsch, R. Grau, S.O. Müller, R. Mayer, et al., Characterization and non-clinical assessment of the proposed etanercept biosimilar GP2015 with originator etanercept (Enbrel®), *Expert Opin. Biol. Ther.* [Internet] 16 (10) (2016) 1185–1195, <https://doi.org/10.1080/14712598.2016.1217329>.
- [5] B. Vezér, Z. Buzás, M. Sebeszta, Z. Zrubka, Authorized manufacturing changes for therapeutic monoclonal antibodies (mAbs) in European Public Assessment Report (EPAR) documents, *Curr. Med Res Opin.* [Internet] 32 (5) (2016) 829–834 (Available from), (<https://www.tandfonline.com/doi/full/10.1185/03007995.2016.1145579>).
- [6] S. Gupta, W. Jiskoot, C. Schöneich, A.S. Rathore, Oxidation and deamidation of monoclonal antibody products: potential impact on stability, biological activity, and efficacy, *J. Pharm. Sci.* [Internet] 111 (4) (2022) 903–918 (Available from), (<https://linkinghub.elsevier.com/retrieve/pii/S0022354921006468>).
- [7] W.C. Lamanna, J. Holzmann, H.P. Cohen, X. Guo, M. Schweigler, T. Stangler, et al., Maintaining consistent quality and clinical performance of biopharmaceuticals, *Expert Opin. Biol. Ther.* [Internet] 18 (4) (2018) 369–379, <https://doi.org/10.1080/14712598.2018.1421169>.
- [8] R.R. Raghavan, R. McCombie, ICH Q5E comparability of biotechnological/biological products subject to changes in their manufacturing processes, *ICH Qual. Guide* (2017) 409–432.
- [9] Agency E.M. June 2005 CPMP / ICH / 5721 / 03 ICH Topic Q 5 E Comparability of Biotechnological / Biological Products Step 5 NOTE FOR GUIDANCE ON BIOTECHNOLOGICAL / BIOLOGICAL PRODUCTS SUBJECT TO CHANGES IN THEIR MANUFACTURING PROCESS (CPMP / ICH / 5721 / 03) TRANSM. 2005; (May 2004). Available from: (<https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-5-e-comparability-biotechnological/biological-product-s-step-5-en.pdf>).
- [10] P. Declerck, M. Farouk-Rezk, P.M. Rudd, Biosimilarity versus manufacturing change: two distinct concepts, *Pharm. Res* 33 (2) (2016) 261–268.
- [11] F. de Mora, Biosimilars: a value proposition, *BioDrugs* [Internet] 33 (4) (2019) 353–356, <https://doi.org/10.1007/s40259-019-00360-7>.
- [12] N. Nupur, S. Joshi, D. Gulliarne, A.S. Rathore, Analytical similarity assessment of biosimilars: global regulatory landscape, *Recent Stud. Major Adv. Orthogonal Platf.* 10 (February) (2022) 1–23.
- [13] S.K. Jung, K.H. Lee, J.W. Jeon, J.W. Lee, B.O. Kwon, Y.J. Kim, et al., Physicochemical characterization of Remsima®, MABs [Internet] 6 (5) (2014) 3) 1163–1177 (Available from), (<http://www.tandfonline.com/doi/full/10.4161/mabs.32221>).
- [14] F. de Mora, B.C.J.M. Fauser, Biosimilars to recombinant human FSH medicines: comparable efficacy and safety to the original biologic (Available from), *Reprod. Biomed. Online* [Internet] 35 (1) (2017) 81–86, <https://doi.org/10.1016/j.rbmo.2017.03.020>.
- [15] Food and Drug Administration. NDA/BLA Multi-disciplinary Review and Evaluation {BLA 103976, Supplement 5231}{Xolair/Xolair} Reference ID: 4326577. September 8, 2017 for initial rollout (NME/original BLA reviews) [Internet]. 2018 [cited 2020 Feb 9]. Available from: (<https://www.fda.gov/meda/124199/download>).
- [16] Fazel R., Guan Y., Vaziri B., Krisp C., Heikaus L., Saadati A., et al. Structural and In Vitro Functional Comparability Analysis of Altebrel™, a Proposed Etanercept Biosimilar: Focus on Primary Sequence and Glycosylation.:1–16.
- [17] S. Fekete, A. Gassner, S. Rudaz, J. Schappeler, Analytical strategies for the characterization of therapeutic monoclonal antibodies (Available from), *Trends Anal. Chem.* [Internet] 42 (2013) 74–83, <https://doi.org/10.1016/j.trac.2012.09.012>.
- [18] Pelaia C., Calabrese C., Terracciano R., Blasio F.De, Vatrella A., Pelaia G. Omalizumab, the first available antibody for biological treatment of severe asthma: more than a decade of real-life effectiveness, 2018; 1–16.
- [19] L.F. Pennington, S. Tarchevskaya, D. Brigger, K. Sathiyamoorthy, M.T. Graham, K. C. Nadeau, et al., omalizumab-mediated IgE exchange (Available from), *Nat. Commun.* [Internet] 7 (May) (2016) 1–12, <https://doi.org/10.1038/ncomms11610>.
- [20] European Medicines Agency (EMA). Guideline on Comparability of Biotechnology-derived medicinal products after a change in the Manufacturing Process - Non-Clinical and Clinical Issues [Internet], 2007. Available from: (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-comparability-biotechnology-derived-medicinal-products-after-change-manufacturing-process_en.pdf).
- [21] European Medicines Agency (EMA). EMA/CHMP/BWP/247713/2012 - Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues (revision 1) 2014, Julio 2020.
- [22] European Medicines Agency. Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues (EMA/CHMP/BWP/42832/2005) [Internet]. 2006. Available from: (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-similar-biological-medicinal-products-containing-biotechnology-derived-proteins-active_en-1.pdf).
- [23] M. Malm, R. Saghaleyni, M. Lundqvist, M. Giudici, V. Chotteau, R. Field, et al., Evolution from adherent to suspension: systems biology of HEK293 cell line development, *Sci. Rep.* [Internet] 10 (1) (2020) 1–15, <https://doi.org/10.1038/s41598-020-76137-8>.
- [24] A.S. Kirshenbaum, Y. Yin, J. Bruce Sundstrom, G. Bandara, D.D. Metcalfe, Description and characterization of a novel human mast cell line for scientific study, *Int. J. Mol. Sci.* 20 (22) (2019) 1–9.
- [25] D. Wan, F. Ludolf, D.G.W. Alanine, O. Stretton, E. Ali Ali, N. Al-Barwary, et al., Use of Humanised Rat Basophilic Leukaemia Cell Line RS-ATL8 for the Assessment of Allergenicity of Schistosoma mansoni Proteins, *PLoS Negl. Trop. Dis.* 8 (9) (2014).
- [26] B.A. Helm, I. Sayers, A. Higginbottom, D.C. Machado, Y. Ling, K. Ahmad, et al., Identification of the high affinity receptor binding region in human immunoglobulin E (Available from), *J. Biol. Chem.* [Internet] 271 (13) (1996) 7494–7500, <https://doi.org/10.1074/jbc.271.13.7494>.
- [27] Kuehn H.S., Radinger M., Gilfillan A.M. Measuring Mast Cell Mediator Release, 2010;1–9.
- [28] D. Dombrowicz, A.T. Brini, V. Flamand, E. Hicks, J.N. Snouwaert, J.P. Kinet, et al., Anaphylaxis mediated through a humanized high affinity IgE receptor, *J. Immunol.* [Internet] 157 (4) (1996) 1645–1651 (Available from), (<http://www.ncbi.nlm.nih.gov/pubmed/8759751>).
- [29] P. Jardié, L.G. Presta, Nucleic acid encoding anti-ige antibodies [Internet]. U. S. ; US 7,351,816 B2 Vol. 2 (2008) (Available from), (<https://patentimages.storage.googleapis.com/2d/a9/c8/d80a93e27d9e6d/US7351816.pdf>).
- [30] L. Hajba, Á. Szekrényes, B. Borza, A. Guttman, On the glycosylation aspects of biosimilarity, *Drug Discov. Today* [Internet] 23 (3) (2018) 616–625, <https://doi.org/10.1016/j.drudis.2018.01.009>.
- [31] M. Kokkinidis, N.M. Glykos, V.E. Fadoulglou, Catalytic activity regulation through post-translational modification: the expanding universe of protein diversity [Internet]. Vol. 122. *Advances in Protein Chemistry and Structural Biology*, Elsevier Ltd., 2020, pp. 97–125, <https://doi.org/10.1016/b.s.apcsb.2020.05.001>.
- [32] Y.-C. Wang, S.E. Peterson, J.F. Loring, Protein post-translational modifications and regulation of pluripotency in human stem cells, *Cell Res* [Internet] 24 (2) (2014) 12) 143–160 (Available from), (<http://www.nature.com/articles/cr2013151>).
- [33] M. Szabo, C. Filep, M. Nagy, D. Sarkozy, M. Szigeti, E. Sperling, et al., N-glycosylation structure – function characterization of omalizumab, an anti-asthma biopharmaceutical product (Available from), *J. Pharm. Biomed. Anal.* [Internet] 209 (2022), 114483, <https://doi.org/10.1016/j.jpba.2021.114483>.
- [34] A. Croset, L. Delafosse, J.P. Gaudry, C. Arod, L. Glez, C. Losberger, et al., Differences in the glycosylation of recombinant proteins expressed in HEK and CHO cells, *J. Biotechnol.* [Internet] 161 (3) (2012) 336–348, <https://doi.org/10.1016/j.jbiotec.2012.06.038>.
- [35] C. Metcalfe, T. Dougall, C. Bird, P. Rigby, M.E. Behr-Gross, M. Wadhwa, et al., The first World Health Organization International Standard for infliximab products: A step towards maintaining harmonized biological activity, MABs [Internet] 11 (1) (2019) 13–25, <https://doi.org/10.1080/19420862.2018.1532766>.
- [36] J.M. Harris, R. Maciua, M.S. Bradley, C.R. Cabanski, H. Scheerens, J. Lim, et al., A randomized trial of the efficacy and safety of quilizumab in adults with inadequately controlled allergic asthma, *Respir. Res* [Internet] 17 (1) (2016) 1–11, <https://doi.org/10.1186/s12931-016-0347-2>.
- [37] M. Maurer, S.S. Saini, K. McLendon, P. Wabnitz, S. Kim, K. Ahn, et al., Pharmacokinetic equivalence of CT-P39 and reference omalizumab in healthy individuals: A randomised, double-blind, parallel-group, Phase 1 trial, *Clin. Transl. Allergy* 12 (11) (2022).