Title: Identification of an Antigenic Determinant of Clavulanic Acid Responsible of IgE-mediated Reactions

Short title: Clavulanic acid determinant in IgE reactions.

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Author contributions:

NB, RFS, AMS performed experiments and analyzed data.
MJT, MS and GB phenotyped the patients and controls.
FN performed molecular modeling and critically read the manuscript.
CM, MJT, EPI and DPS critically read the manuscript and provided insightful information and discussion valuable for design and interpretation of data.
TDF, MIM and MJT designed the study, analyzed data and wrote the manuscript.

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NB, CM, MS, FN, EPI, TDF, MIM and MJT have a patent related with this paper (P201631133)

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Abstract.

Background: Selective reactions to clavulanic acid (CLV) account for around 30% of immediate reactions after administration of amoxicillin-CLV. Currently, none immunoassay is available for detecting specific IgE to CLV, and its specific recognition in patients with immediate reactions has only been demonstrated by basophil activation testing. The lack of knowledge regarding the structure of the drug that remains bound to proteins (antigenic determinant) is hampering the development of *in vitro* diagnostics. We aimed to identify the

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antigenic determinants of CLV as well as to evaluate their specific IgE recognition and potential role for diagnosis.

Methods: We hypothesized the formation of two antigenic determinants for CLV, AD-I (*N*-protein, 3-oxopropanamide) and AD-II (*N*-protein, 3-aminopropanamide), and designed different synthetic analogs to each one. IgE recognition of these structures was evaluated in basophils from patients with selective reactions to CLV and tolerant subjects. In parallel, the CLV fragments bound to proteins were identified by proteomic approaches.

Results: Two synthetic analogs of AD-I were found to activate basophils from allergic patients. This determinant was also detected bound to lysines 195 and 475 of CLV-treated human serum albumin. One of these analogs was able to activate basophils in 59% of patients whereas CLV only in 41%. Combining both results led to an increase in basophil activation in 69% of patients, and only in 12% of controls.

Conclusion: We have identified AD-I as one of the antigenic determinants of CLV, which is the drug fragment that remains protein-bound and can be recognized by specific IgE on basophils.

Key words.

Basophils; clavulanic acid; drug allergy; IgE; in vitro test.

Abbreviations.

AX: Amoxicillin

BAT: Basophil activation test

CLV: Clavulanic acid

EAACI: European Academy of Allergy and Clinical Immunology

ENDA: European Network for Drug Allergy

HSA: Human Serum Albumin

LC-MS/MS: Liquid chromatography-Mass spectrometry

SI: Stimulation index

WTM: Wortmannin

INTRODUCTION

β-lactam antibiotics are the drugs most frequently involved in drug allergic reactions due to their high consumption (1). Increasing bacterial resistance against these antibiotics is a global health challenge (1). This has prompted the search for novel inhibitors of β-lactamases against a wide variety of gram-positive and gram-negative bacteria, such as clavulanic acid (CLV) (2, 3). The immunogenicity of this inhibitor, prescribed along with amoxicillin (AX), has been overlooked based on initial studies (4). First immediate selective reactions to CLV were described in 1995 (5), and reports of these reactions have increased in the last decades (6, 7), accounting nowadays for 30% of all reactions to AX-CLV (6).

As a consequence of increasing allergic reactions to CLV, there is much interest in elucidating the underlying mechanisms and structures involved in its immunological recognition (8, 9). As other β-lactams, CLV must bind covalently to carrier proteins to form conjugates with sufficient size to be recognized by the immune system and induce a response (9). Human serum albumin (HSA) can be considered the main protein target for haptenation by β-lactams, since it is the most abundant protein in plasma, has multiple binding sites, and acts as a carrier for endogenous and exogenous compounds (10). To gain insight into the haptenation process, proteomic studies with HSA have been performed with different β-lactams (11-16). Conjugation of these antibiotics is mainly driven by the nucleophilic attack of lysine amino groups from the protein to the electrophilic carbonyl group of the β-lactam ring (9, 10). The resultant conjugates contain the antigenic determinants that are recognized by specific IgE; these may include part of the carrier protein and the drug. However, the chemical structures of the CLV antigenic determinants are still unknown, due to its complex and unpredictable reactivity. CLV chemical structure consists of a bicyclic ring system quite different from other β-lactams (17). It is thought the substitution of a sulfur atom by an oxygen atom (oxazolidine instead of thiazolidine ring) increases strain on the bicyclic system, making it more reactive towards nucleophiles. Additional structural differences underlie the unexpected reactivity of CLV and the high instability of its derived structures (17-20).

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Protein haptenation by CLV is thought to occur similarly to other β-lactams (4, 10). The reaction of lysine residues with CLV opens the strained β-lactam ring, leading to the formation of acylprotein [1]. However, due to its high instability, the resulting structure rapidly degrades, leading to the formation of multiple fragments (Figure 1A): Acylprotein [1] undergoes the subsequent opening of the five-member oxazolidine ring, forming the linear imine [2]. This intermediate [2] may follow different reactivity pathways: a) decarboxylation giving rise to derivative [3], which like molecule [2], can break down via the imine bond to yield the protein conjugated to the derived aldehyde (4, 8, 9), which we hypothesize as a possible antigenic determinant (hereinafter called AD-I); b) isomerization of both intermediates [2] and [3] to the imine [5], which would be hydrolyzed under biological conditions leading to the formation of the protein conjugated to the 3-aminopropanamide, forming a second possible determinant that we have named AD-II; and c)

isomerization to the corresponding enamine [4], which apart from decarboxylation, in principle, should not experience further fragmentation.

The two main proposed antigenic determinants of CLV, AD-I and AD-II, consist of very low molecular weight structures formed by only 3 carbon atoms. Whereas AD-I (1,3-dicarbonylic compound) may be fairly reactive towards an additional nucleophilic attack of amino groups of proteins through its terminal aldehyde functional group, reactivity of AD-II appears quite limited. It should be pointed out that the rest of the described intermediates (molecules [1] to [5] of Figure 1A) could also be possible antigenic determinants involved in IgE recognition. It is assumed that different chemical functional groups would influence the molecular recognition and immunogenicity of compounds. The analysis of structure–activity relationship would enable the determination of the chemical groups responsible for CLV recognition by the immune system. This strategy has been successfully employed for studying the involvement of drug metabolites or derived structures in IgE molecular recognition (21, 22). Moreover, the identification of the most relevant antigenic determinants of CLV could help improve current diagnostic procedures.

Diagnosis work-up of CLV allergy usually includes a clinical history, skin test and drug provocation test (6, 23). However, clinical history is often unreliable, moreover the sensitivity of skin tests to CLV is not optimal and is only available in some countries (9). Consequently, drug provocation testing must be performed in most cases to establish diagnosis. This procedure cannot be performed with CLV itself, but indirectly using both AX and AX-CLV. Therefore, diagnosis of CLV allergy must be made by assessing tolerance to AX in patients with positive drug provocation test results to AX-CLV (9). These *in vivo* tests are not risk-free and are both time consuming and expensive. *In vitro* tests can be useful for β-lactam diagnosis (24), however for CLV, only the cellular tests, basophil activation test (BAT) (6, 25) and histamine release test (26) show potential. Nevertheless, the sensitivity of these *in vitro* assays using CLV itself is still not good enough and diagnosis cannot be confirmed for an important percentage of patients. This could be due to the fact that the correct antigenic determinants that acted as the first sensitizers are not included in the test. In this vein, a previous study demonstrated that the inclusion of different pyrazolone metabolites in BAT was a successful approach to increase sensitivity (22), a fact that could be extrapolated to CLV.

In order to identify the antigenic determinants better recognized by the immune system we developed synthetic analogs corresponding to the two hypothesized antigenic determinants of CLV (AD-I and AD-II). Subsequently we evaluated their capacity to activate basophils in a well characterized group of patients. Moreover, we investigated whether the specifically recognized antigenic determinant corresponds to the resulting structure of the CLV that remains linked to the protein.

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METHODS

Patients.

This study included 29 patients with immediate hypersensitivity reactions to CLV after AX-CLV intake. Diagnosis was confirmed following the European Academy of Allergy and Clinical Immunology (EAACI)/European Network for Drug Allergy (ENDA) guidelines (27). Clinical data and individual results for the allergological work-up are shown in Table S1. As a control group, we selected 25 cases with confirmed tolerance to AX-CLV. The study was conducted according to the Declaration of Helsinki principles and was approved by the Provincial Ethics Committee of Malaga. All subjects included in the study were informed orally and signed the corresponding informed consent.

Methods.

Patients were diagnosed by skin testing or drug provocation test according to the EAACI guidelines (27). Analogs of determinants were synthesized using conventional chemistry methods. BAT was performed as previously described (28) using synthetic analogs and CLV at different concentrations. To confirm IgE-mediated basophil activation, the inhibitory effect of wortmannin was analyzed (29). *In vitro* HSA modification with CLV was studied by MALDI-TOF and liquid chromatography-mass spectrometric (LC-MS/MS) analysis. For the latter, protein conjugate was digested with arginine C, mass spectrometry (Orbitrap) performed for peptide sequencing and protein matching (MASCOT). The crystal structures of HSA and CLV were obtained from RCSB Protein Data Bank (PDB ID: 1AO6 and PDB ID: J01, respectively) (30). The relaxation of the chain-A from HSA was performed with AMBER 12 MD software package (31). Then, for each recognized site of addition, a docking analysis was performed using the AutoDock 4.2 software (32).

For further details, see the Methods section in this article's Online Repository.

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3 172 **RESULTS**
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5 173 **Synthesis of antigenic determinants derived from CLV**
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7 174 Six antigenic determinant analogs were obtained (Figure 1B). Three of these, Clav1, Clav2 and Clav3 were
8 175 prepared to emulate the AD-I structure. In Clav1, both carbonyl groups of the molecule are protected, one as
9 176 an amide and the other as an acetal group, so the expected reactivity is low. However, the carbonyl groups of
10 177 both Clav2 and Clav3 were activated, modified chemically to form the anhydride, to enhance their reactivity
11 178 towards nucleophiles. The main difference is that Clav3 has one of the carbonyl groups protected as an acetal,
12 179 diminishing its ability to bind to proteins compared to Clav2. The other three structures, Clav4, Clav5 and Clav6
13 180 were chosen as model analogs of AD-II. Clav4 and Clav5, are less reactive than Clav6, because their carbonyl
14 181 groups have not been activated. The resulting conjugate of Clav6 would present as an AD-II structure but with
15 182 its amino group protected as an acyl group.
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17 183 **Basophil activation test**
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19 184 All synthetic analogs of CLV determinants and CLV itself were evaluated in patients and controls. The optimal
20 185 concentration to study basophil activation with each structure was selected using dose-response curves
21 186 (Figure 2). Analyzing the expression of CD63, the only concentration of CLV that showed significant differences
22 187 between patients and controls was 8mM (p=0.042) (Figure 2A). For the different synthetic analogs, only Clav2
23 188 and Clav3, both coming from the AD-I, showed significant differences between patients and controls (Figure
24 189 2B). For Clav2 the best concentrations were 4mM (p=0.022) and 8mM (p=0.008), whereas for Clav3 only the
25 190 highest concentration (8mM) showed significant differences (p=0.049). Individual results of BAT expressed as
26 191 stimulation index (SI) are shown in Table S2. Other concentrations higher than 8mM were also tested, but
27 192 they were discarded due to their high cytotoxicity (data not shown). Only the structures that induced basophil
28 193 activation in patients (CLV, Clav2 and Clav3), at the concentration of 8mM, were used for further study.
29
30 194 Finally, to prove that the observed activation was IgE mediated, BAT was performed after incubating cells with
31 195 wortmannin (WTM), a potent inhibitor of the IgE signaling pathway. In all cases, this treatment significantly
32 196 reduced the percentage of CD63 expression compared with BAT performed in the absence of WTM (p<0.001
33 197 for CLV; p<0.001 for Clav2; p=0.007 for Clav3) (Figure 2C).
34
35 198 ROC curves for these structures were performed to select the cut-off to obtain the best sensitivity/specificity
36 199 balance (Figure 3A) and this was established at: SI>2 for CLV; and SI >4 for Clav2 and Clav3 (Figure 3B). Based
37 200 on the chosen cut-off, we observed a positive basophil activation with CLV in 41.4% of patients, with Clav2 in
38 201 58.6% and with Clav3 in 27.6%. In controls, basophil activation was positive in 12% of cases for all three
39 202 structures (Figure 3B).
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We observed that from those patients with positive BAT to CLV, 75% were also positive to Clav2 and 33.3% to Clav3. More interestingly, from cases with negative BAT to CLV, 47.1% showed positive results with Clav2 and 23.5% to Clav3 (Figure 4A).

The percentage of patients showing basophil activation increased significantly to 69.0% when results obtained with CLV and Clav2 were combined compared with CLV alone (p=0.002), however no improvement was observed when Clav3 was added (Figure 4B and Table S2).

***In vitro* studies of HSA modification with CLV**

In view of the results obtained with BAT, and to confirm that this specifically recognized antigenic determinant forms part of the resulting CLV-protein conjugate, we used CLV-treated HSA as a model, and analyzed the conjugates using proteomic. We incubated HSA with different CLV concentrations ranging from those close to physiological conditions (1:10) to a high excess of the drug (1:600). The study of the protein fraction by MALDI-TOF MS showed that incubation of HSA in the presence of increasing concentrations of CLV induced a concentration-dependent increase in the mass of the protein (Figure S2), from 1:10 up to a 1:320 molar ratio. Saturation of CLV binding to HSA occurs at a 1:320 molar ratio, since conjugates derived from a 1:600 molar ratio showed the same mass increment.

Since HSA modifications could be detected with the conjugate derived from a 1:10 molar ratio, we used this for further characterization studies, given that it is the closest to therapeutic conditions. Residues modified by CLV were identified by arginine C digestion of native and modified HSA prepared at neutral pH, followed by LC-MS/MS analysis of the resulting peptides. We observed that, *in vitro*, CLV binds covalently to the amino groups of lysine residues in HSA. The resulting conjugates, formed after β-lactam ring opening, show a mass increase of 70 Da, which is consistent with the addition of a fragment of the CLV molecule of 71 Da accompanied by the loss of a hydrogen atom (Figure 5). This finding is compatible with the haptenation process proposed in Figure 1. Interestingly, mass increments corresponding to the entire molecule of CLV were not found.

When injecting 1µL of conjugate in the LC-MS/MS analyzer, we observed one peptide (^{473}VTK*CCTESLVNR^{484}, calculated mass: 1421.6625 Da) showing a mass increase of 70 Da on Lys 475. The MS/MS fragmentation pathway was carried out from the $[M+2]^{2+}$ as parent ion at m/z 711.8385 Da (Figure 5A). In order to increase the coverage and find additional modified peptides, a higher amount of sample was injected (4µL). This led us to observe a new peptide (^{187}DEGKASSAK*QR^{197}, calculated mass: 1245.5949 Da) covalently modified at Lys 195. Figure 5B shows the MS/MS spectrum for a triply charged ion at m/z 416.2059, corresponding to the peptide HSA 187-197 with the 70 Da mass increment. The peptide sequences were confirmed by the b and y ion series determined by MS/MS. The same peptides were also observed without modification, demonstrating that not all the HSA is modified in those residues at therapeutic dose conditions. It should also be noted that the conditions at which the MS/MS experiments took place, performed in collision induced dissociation (CID)

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237 mode, could cause fragmentation due to the unstable nature of this modification, which could be lost during
238 the collision.

239 **Molecular Modeling**

240 In order to obtain further insights into the observed adducted lysine residues (Lys 195 and Lys 475), molecular
241 modeling studies were performed, exploring the regions surrounding the modified residues. Even though Lys
242 475 is more basic than Lys 195, the former is more exposed because it is located at the surface of HSA, enabling
243 CLV to get close. Lys 195, although less accessible, is surrounded by other positively charge residues that
244 contribute a reduced basicity (12).

245 Docking simulations were used to know how the region adjacent to the adducted Lys residues can stabilize an
246 appropriate complex with CLV and contribute to adduct formation. Figure 6 shows these complexes with the
247 interactions stabilizing CLV in the proximity of Lys 195 or Lys 475. In both complexes, the CLV is visibly close
248 and with the proper orientation to react with the adducted lysines. The nitrogen atom in the lysine residues
249 reacts with the carbon atom of the carbonyl group of the β-lactam ring. In both cases, the carboxylate group
250 of CLV is involved in a salt bridge (with Arg 218 for Lys 195 and Arg 472 for Lys 475) which plays a key role in
251 the orientation of CLV. In the case of Lys 195, an additional hydrogen bond between the hydroxyl of CLV and
252 His 242 also contributes to stabilizing the complex.

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DISCUSSION

Allergic reactions to CLV are rising due to the increasing consumption of AX-CLV (9). Determining which of these two drugs is responsible for a given reaction remains a challenge. This has important therapeutic consequences, because CLV allergic patients can tolerate other β-lactams, including AX (6). *In vitro* tests, such as BAT (6, 25) and the histamine release test (26), have been used for evaluating these patients using whole CLV, however they show sub-optimal sensitivity. Improving existing approaches for diagnosing CLV allergy and developing new ones requires the identification of CLV determinants recognized by the immune system.

Compared to penicillins, for which several antigenic determinants have been characterized, CLV reactivity is very complex and degradation pathways after its conjugation to proteins may lead to multiple possible determinants (8, 9). We have hypothesized the formation of two main antigenic determinants (AD-I and AD-II) in conditions similar to those occurring in the clinical practice, based on preliminary reported data (4, 8) and the chemical reactivity of CLV (17-20, 33-35).

Conjugation of CLV with proteins is assumed to occur through covalent binding of the amino groups of lysine residues in proteins to the carbonyl β-lactam ring, leading to the acyl-protein intermediate 1 (Figure 1A). This is a highly unstable molecule that should undergo rapid oxazolidine ring opening to generate a linear imine intermediate 2. This subsequent structure could itself undergo different reactions, leading to heterogeneous epitopes with a very low density in the carrier. The most representative ones are illustrated in Figure 1A, including the aldehyde derived AD-I and the amine-functionalized AD-II.

AD-I and AD-II consist of small structures (71 Da or 72 Da, respectively), considered too small to be immunogenic. Thus, their corresponding analogs were designed either including butylamide that emulates the linkage to the protein (Clav1 and Clav4) or with ability to bind proteins during the cellular assay (Clav2, Clav3, Clav5 and Clav6) (Figure 1B). From these, Clav2, Clav3 and Clav6 were designed with greater reactivity against proteins.

We evaluated the ability of these structures to stimulate basophils, finding that only two of them (Clav2 and Clav3) were able to induce activation in allergic patients. Interestingly, both molecules correspond to AD-I, suggesting that these synthetic analogs, containing aldehyde functionality, are the ones recognized by IgE bound on the basophil surface. Moreover, the hydrolysis of the second carbonyl group of Clav2 after protein conjugation would give rise to a molecule that is more similar to AD-I than Clav3, which is in agreement with their degree of recognition (Clav2 > Clav3). Basophil activation requires the crosslinking of two adjacent IgEs at the cell surface, and this is only possible with the recognition of a structure with a size ranging from 40-100 Å (36). This explains the fact that in our study only those analogs able to bind proteins can stimulate the basophil. This is in agreement with the hapten hypothesis, which is currently accepted as the mechanism of action for other β-lactams, such as penicillins (10, 37). The IgE-mediated mechanism was confirmed by inhibition of the basophil activation with WTM, an inhibitor of phosphoinositide 3-kinase enzymes, which are

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287 part of the IgE signaling pathway (29) for FcεRI receptor cross-linking (38). Although Clav6 can also bind to
288 proteins, its conjugates are not recognized by IgE from CLV allergic patients. This suggests that small
289 differences in the structure can highly influence the immunological recognition.

290 We evaluated whether the inclusion of these synthetic analogs can improve the potential of BAT for
291 diagnosing CLV allergic patients. Using the complete molecule of CLV, 41.4% of patients showed basophil
292 activation, whereas using Clav2, this increased to 58.6%. Interestingly, the combination of results obtained
293 with CLV and Clav2 significantly increased the percentage of patients showing basophil activation to 69.0%,
294 due to a proportion of patients that were activated by only one molecule. When CLV is used for the assay, the
295 resulting conjugates can contain different and heterogeneous determinants, including the ones we proposed
296 as main determinants (AD-I and AD-II) and others (Fig 1A). Thus, the concentration of AD-I would be lower
297 than the one that we used *in vitro* when we included the synthetic analog Clav2. This lower density of AD-I
298 would affect the recognition in BAT and could explain why some patients that respond to Clav2 do not respond
299 to CLV. A similar pattern has been observed with metamizole in BAT studies: some patients showing IgE
300 recognition to some of its metabolites did not recognize the parent drug (22). BAT combining metamizole plus
301 metabolites results showed an increase of positivity compared to metamizole alone. All patients that
302 responded to metamizole also responded to any of its metabolites, however in the present study we have
303 found some patients that respond to CLV and not to Clav2. These patients may be sensitized to other
304 determinants formed during CLV conjugate fragmentation, which have not been considered in the present
305 study.

306 The formation of hapten-protein conjugates is a key process in β-lactam allergic reactions. In the case of CLV
307 as a hapten, neither the chemical structure of its antigenic determinant or the relevance of the proteins
308 involved is known. The role of the protein is crucial to understand immediate reactions to this drug and their
309 underlying mechanisms. To gain insight into the nature of the relevant determinants formed, we investigated
310 the protein conjugation process using HSA as a model. We observed that covalent binding of CLV to HSA takes
311 place *in vitro* and that this modification is concentration-dependent, consistent with recent studies (8). The
312 identification of the sites modified by CLV in HSA was carried out only for the conjugate formed with the lower
313 concentration of CLV (1:10 molar ratio), in conditions similar to those of therapeutic use. Similar studies
314 carried out for several penicillins (benzylpenicillin, AX, flucoxacillin, piperacillin) showed their corresponding
315 penicilloyl determinant as the only antigenic determinant, stable structure that consists of the opening of the
316 β-lactam ring (12). In contrast, and as expected given the complexity of CLV reactivity, analysis of the CLV-
317 protein conjugates did not allow the detection of mass increments corresponding to the addition of the
318 complete CLV molecule, and only a 70 Da mass increase was observed, corresponding to the incorporation of
319 a 71 Da fragment with loss of a hydrogen atom. Interestingly, this mass increment corresponds to an adduct
320 with AD-I structure, which is in agreement with one fragment recently identified in cell culture media and in
321 patients exposed to AX-CLV with drug induced liver injury (8). Moreover, these results are also consistent with

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3 322 mechanisms and structures proposed in other publications out of the context of allergy, describing CLV
4 323 binding to β-lactamases (17-19), although through a serine residue rather than a lysine (9).

324 This modification of HSA with AD-I was observed at two different lysine residues, Lys 195 and Lys 475, after 325 incubation at conditions close to clinical settings. Moreover, modeling studies were able to explain the 326 reactivity of these residues which is determined by the surrounding environment and residue accessibility. Lys 327 195 has been previously reported to be linked *in vitro* to a bigger CLV-derived structure with a mass equivalent 328 to three AD-I moieties (8). This result can be attributed to the self-condensation of CLV degradation molecules 329 (71 Da) at higher drug concentrations. The same residue has been shown to be modified by β-lactams, such 330 as benzylpenicillin (15), flucoxacillin (16) and piperacillin (14). Lys 475 has not been previously reported to be 331 modified by CLV, although it has been identified as target for AX (8).

332 In conclusion, we have studied the relationship between the chemical structure of different potential 333 determinants of CLV and their immunological recognition. We have proposed the molecules AD-I and AD-II as 334 potential antigenic determinants. Considering the structure-activity relationship of these determinants, it 335 appears that the existence of a carbonyl functional group in AD-I instead of an amino group as happens in 336 AD-II may increase its immunogenicity. Moreover, another necessary factor for these determinants to be 337 recognized by the immune system seems to be their capacity to bind to proteins. A synthetic analog with both 338 a carbonyl group and capacity to bind proteins, Clav2, has been shown to activate basophils from CLV allergic 339 patients, to higher degree than CLV itself, and thus represents a promising lead to improve the sensitivity of 340 *in vitro* diagnosis. Importantly, the capacity of AD-I fragments to bind HSA, forming adducts, has also been 341 confirmed by proteomic studies. These findings will help us improve current tests and develop new diagnostic 342 tools, such as immunoassays, which will aid clinicians when performing an allergological work-up.

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FIGURE CAPTIONS

Figure 1. Hypothesized mechanisms for protein haptenation with CLV. **A**, Proposed reaction mechanism for covalent binding of CLV with protein and different fragmentation pathways. **B**, Synthetic analogs corresponding to AD-I (Clav1-Clav3) and AD-II (Clav4-Clav6).

Figure 2. Evaluation of BAT performed with different structures related to CLV. **A**, Dose-response curve for BAT using CLV. **B**, Dose-response curves for BAT using synthetic analogs (Clav1-Clav6). **C**, Results of the inhibition of basophil activation with wortmannin (WTM) after stimulation with CLV, Clav2 and Clav3 or positive control (anti-IgE). Bars show the mean value + SD of percentage of CD63 obtained for non-inhibited and WTM inhibited basophil activation. Results are expressed as the mean + SD of %CD63 in patients (N = 29) and healthy controls (N = 25). Significant differences are indicated in the graph (* $p<0.05$ and ** $p<0.01$).

Figure 3. Determination of BAT cut-off for CLV, Clav2 and Clav3 at 8 mM concentration. **A**, ROC curve analysis of each structure. Red arrows represent the SI value showing the best sensitivity/specificity balance. **B**, Dot plots graphs showing individual BAT results expressed as SI of each structure for patients and controls. Positive results for BAT (over cut-off) in allergic patients and controls are represented as percentages.

Figure 4. Positive BAT results in allergic patients. **A**, Comparison of the percentage of positive results of BAT for Clav2 and Clav3 in cases with positive or negative BAT to CLV. **B**, Comparison of the percentage of positivity of BAT combining the results with CLV, Clav2 or Clav3. Significant differences in comparison with CLV alone are indicated in the graph (* $p<0.01$).

Figure 5. MS/MS spectra of CLV-HSA derived peptides identified *in vitro* with a mass increment of 70 Da. **A**, Collision-induced dissociation (CID) based MS/MS spectra of the $[M+2H]^{2+}$ precursor ion of the peptide ^{473}VTK(+70)CCTESLVNR^{484} modified at Lys 475. Calculated mass of modified peptide: 1421.6643, experimental mass: 1421.6625 and observed parent ion at *m/z*: 711.8385. **B**, Collision-induced dissociation (CID) based MS/MS spectra of the $[M+3H]^{3+}$ precursor ion of the peptide ^{187}DEGKASSAK(+70)QR^{197} modified at Lys 195. Calculated mass of modified peptide: 1245.5949, experimental mass: 1245.5960 and observed parent ion at *m/z*: 416.2059. Matched b- and y-ions are indicated in the spectra as well as the peptide sequence.

Figure 6. Main interactions stabilizing CLV in the surrounding of adducted lysines. **A**, Lys 195. **B**, Lys 475. The residues implied in the stabilization and orientation of CLV are shown.

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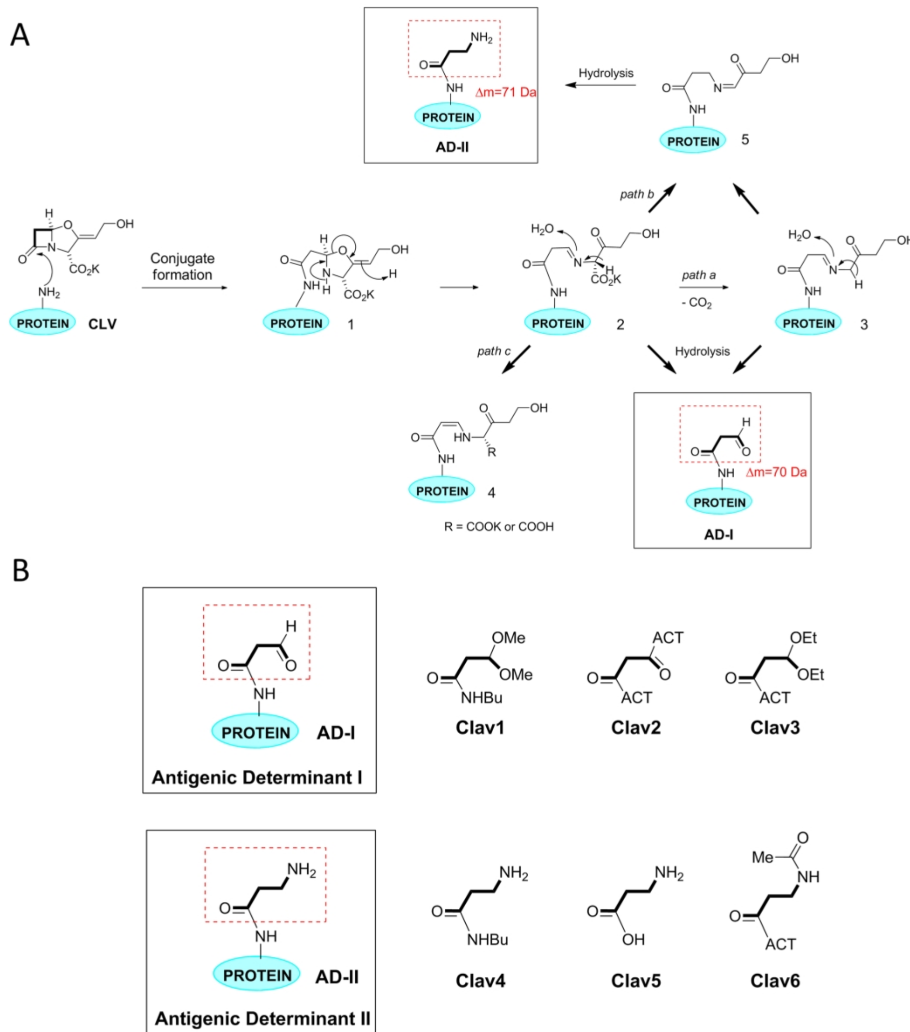

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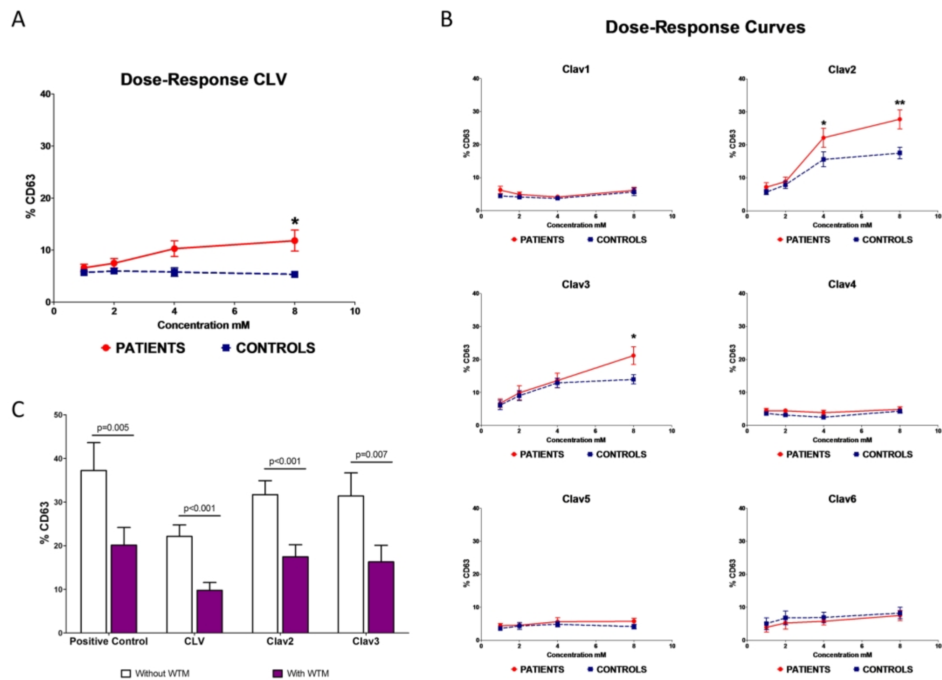

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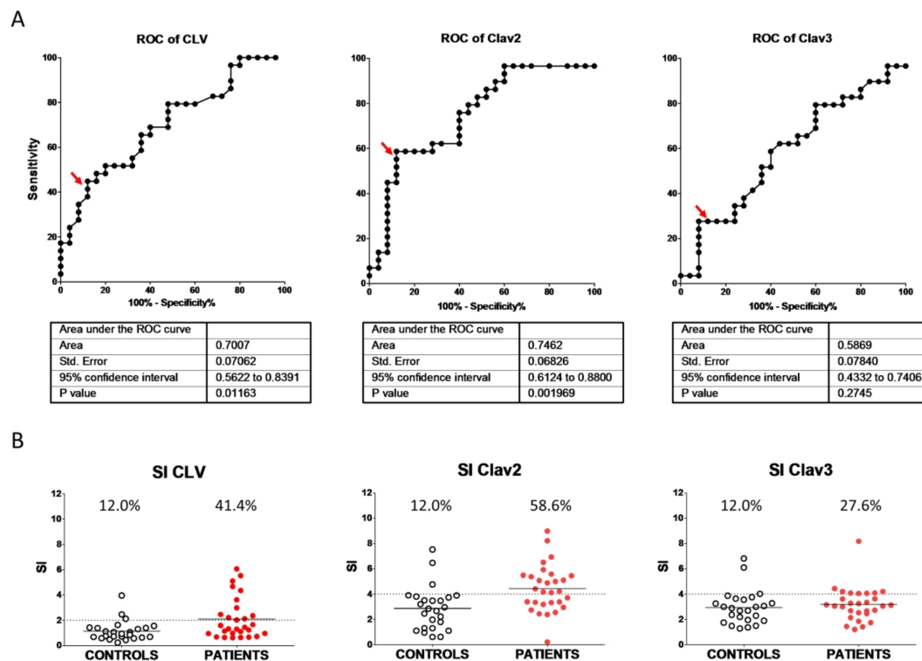

ROC of CLV

Area under the ROC curve	
Area	0.7007
Std. Error	0.07062
95% confidence interval	0.5622 to 0.8391
P value	0.01163

ROC of Clav2

Area under the ROC curve	
Area	0.7462
Std. Error	0.06826
95% confidence interval	0.6124 to 0.8800
P value	0.001969

ROC of Clav3

Area under the ROC curve	
Area	0.5869
Std. Error	0.07840
95% confidence interval	0.4332 to 0.7406
P value	0.2745

Determination of BAT cut-off for CLV, Clav2 and Clav3 at 8 mM concentration. A, ROC curve analysis of each structure. Red arrows represent the SI value showing the best sensitivity/specificity balance. B, Dot plots graphs showing individual BAT results expressed as SI of each structure for patients and controls. Positive results for BAT (over cut-off) in allergic patients and controls are represented as percentages.

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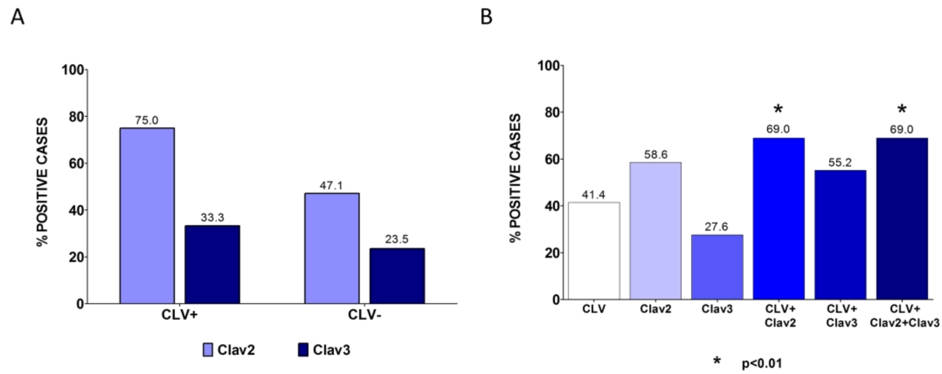

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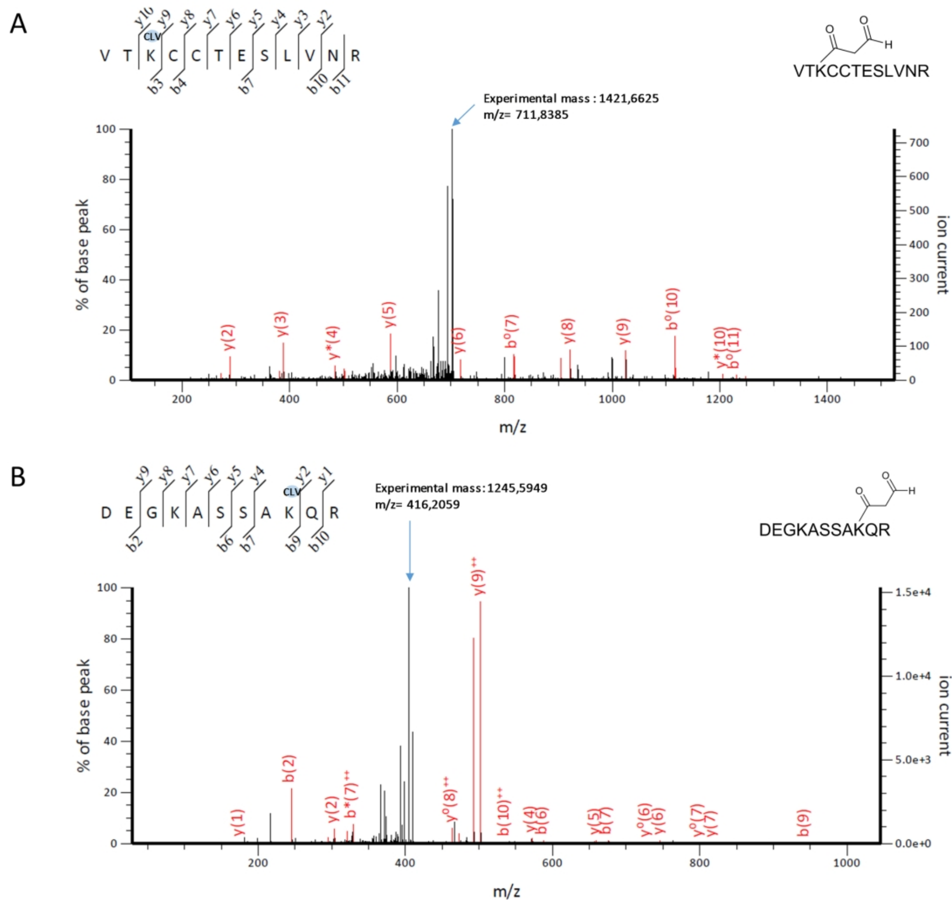

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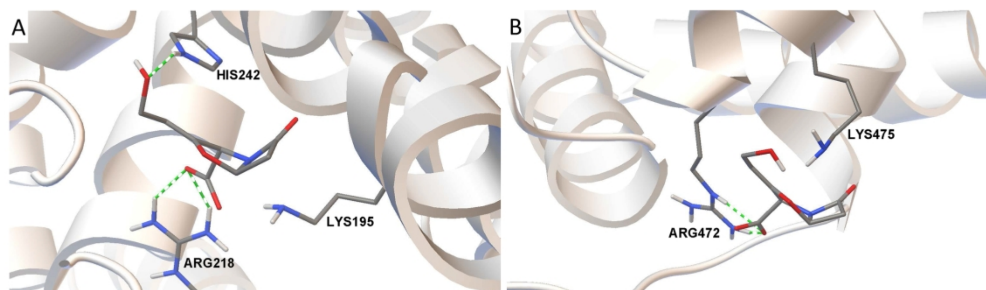

Main interactions stabilizing CLV in the surrounding of adducted lysines. A, Lys 195. B, Lys 475. The residues implied in the stabilization and orientation of CLV are shown.

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