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Characterization of anaphylaxis reveals different metabolic changes depending on severity and triggers

Carolina Perales-Chorda[1*] | David Obeso[2,3*] | Laura Twomey[2,3] | Ayelén Rojas-Benedicto[4] | Leonor Puchades-Carrasco[4] | Marta Roca[5] | Antonio Pineda-Lucena[4,6] | José Julio Laguna[7,8] | Coral Barbas[3] | Vanesa Esteban[8,9] | Jaume Martí-Garrido[1] | Ethel Ibañez-Echevarria[1] | Ramón López-Salgueiro[1] | Domingo Barber[2] | Alma Villaseñor[2*] | Dolores Hernández Fernández de Rojas[1*]

[1]Allergy Department of Hospital, Universitari i Politècnic La Fe, Valencia, Spain

[2]IMMA, Instituto de Medicina Molecular Aplicada, Departamento de Ciencias Médicas Básicas, Facultad de Medicina, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe| Boadilla del Monte, Madrid, 28660, Spain

[3]CEMBIO, Centre for Metabolomics and Bioanalysis, Department of Chemistry and Biochemistry, Facultad de Farmacia, Universidad San Pablo-CEU, CEU Universities, Urbanización Montepríncipe| Boadilla del Monte, Madrid, 28660, Spain

[4]Drug Discovery Unit, Health Research Institute La Fe, Valencia, Spain

[5]Analytical Unit, Health Research Institute Hospital La Fe, Valencia, Spain

[6]Molecular Therapeutics Program, Center for Applied Medical Research, University of Navarra, Pamplona, Spain

[7]Allergy Unit, Allergo-Anaesthesia Unit, Hospital Central de la Cruz Roja, Madrid, Spain

[8]Faculty of Medicine and Biomedicine, Alfonso X El Sabio University, Madrid, Spain

[9]Department of Allergy and Immunology, IIS-Fundación Jiménez Díaz, UAM, Madrid, Spain

Correspondence
Dolores Hernández Fernández de Rojas, MD, PhD, Allergy Research Group - IIS - Hospital Universitari i Politècnic La Fe, Avinguda de Fernando Abril Martorell, 106, 46026 València, Valencia, España.
Email: hernandez_dol@gva.es

Alma Villaseñor, PhD, Department of Basic Medical Sciences, Faculty of Medicine. San Pablo CEU University, Campus Montepríncipe. Crtra. Boadilla del Monte km 5.3., CP 28668 Boadilla del Monte. Madrid, Spain
Email: alma.villasenor@ceu.es

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Abstract

Background: Despite the increasing incidence of anaphylaxis, its underlying molecular mechanisms and biomarkers for appropriate diagnosis remain undetermined. The rapid onset and potentially fatal outcome in the absence of managed treatment prevent its study. Up today, there are still no known biomarkers that allow an unequivocal diagnosis. Therefore, the aim of this study was to explore metabolic changes in patients suffering anaphylactic reactions depending on the trigger (food and/or drug) and severity (moderate and severe) in a real-life set-up.

Methods: Eighteen episodes of anaphylaxis, one per patient, were analysed. Sera were collected during the acute phase (T1), the recovery phase (T2) and around 2–3 months after the anaphylactic reaction (T0: basal state). Reactions were classified following an exhaustive allergological evaluation for severity and trigger. Sera samples were analysed using untargeted metabolomics combining liquid chromatography coupled to mass spectrometry (LC-MS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR).

*These authors have equally contributed.

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Results: 'Food T1 *vs* T2' and 'moderate T1 *vs* T2' anaphylaxis comparisons showed clear metabolic patterns during the onset of an anaphylactic reaction, which differed from those induced by drugs, food + drug or severe anaphylaxis. Moreover, the model of food anaphylaxis was able to distinguish the well-characterized IgE (antibiotics) from non-IgE-mediated anaphylaxis (nonsteroidal anti-inflammatory drugs), suggesting a differential metabolic pathway associated with the mechanism of action. Metabolic differences between 'moderate *vs* severe' at the acute phase T1 and at basal state T0 were studied. Among the altered metabolites, glucose, lipids, cortisol, betaine and oleamide were observed altered.

Conclusions: The results of this exploratory study provide the first evidence that different anaphylactic triggers or severity induce differential metabolic changes along time or at specific time-point, respectively. Besides, the basal status T0 might identify high-risk patients, thus opening new ways to understand, diagnose and treat anaphylaxis.

KEYWORDS
^{1}H-NMR, drug anaphylaxis, food anaphylaxis, grading anaphylaxis, IgE-mediated anaphylaxis, untargeted metabolomics, UPLC-MS

1 | INTRODUCTION

Anaphylaxis is a serious systemic hypersensitivity reaction that is usually rapid in onset and may cause death.[1] Its incidence is estimated to be between 50 and 103 cases per 100,000 person-years. Though the mortality rate has remained stable, there has been an increase in the number of hospital admissions due to anaphylaxis of up to 7 times in the last 10 years.[2] From a clinical point of view, it is a complex syndrome that can involve multiple organs, including the skin and those of the digestive, respiratory, nervous and cardiovascular systems.[3]

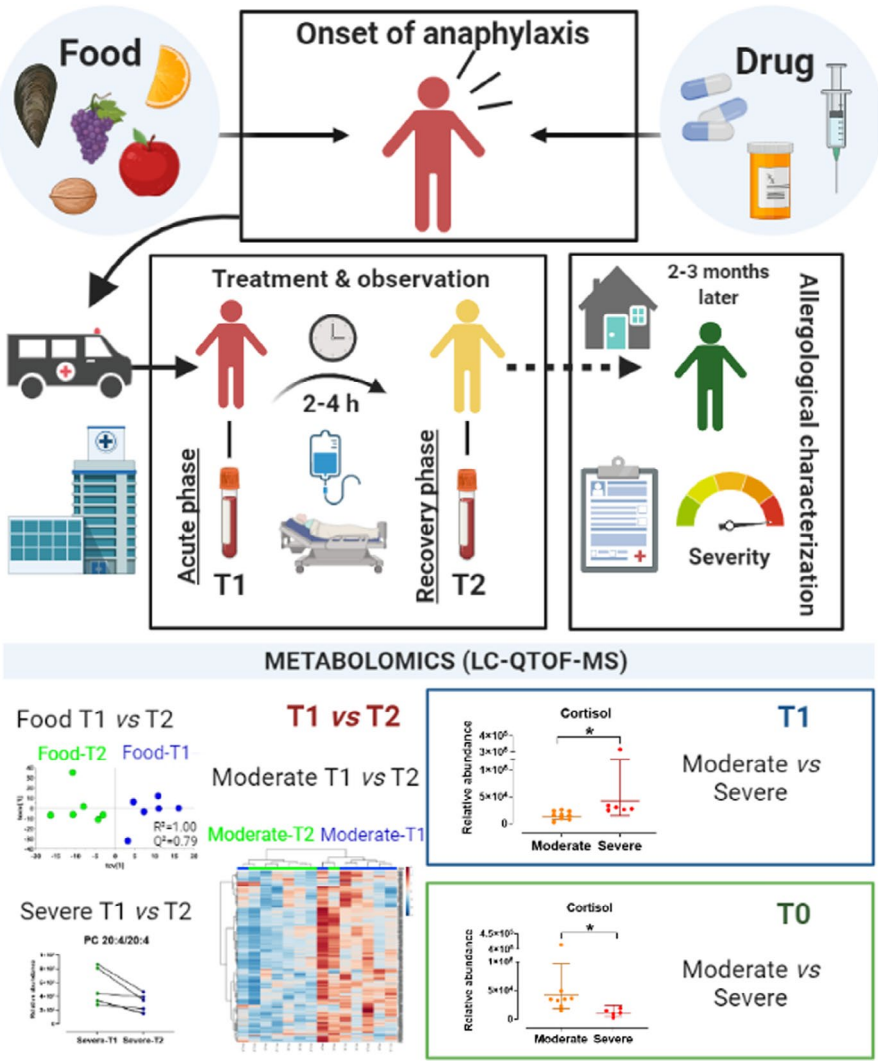
Although potentially any substance can cause an anaphylactic reaction, the most common aetiological agents in adults are drugs, foods and hymenoptera stings. The allergen triggers the release of chemical mediators by the effector cells (mainly mast cells and basophils), which cause the symptoms.[4,5] The nature of the allergen determines the molecular mechanism by which mediators are released: dependent or independent of immunoglobulin E (IgE). IgE-dependent anaphylaxis has been clearly characterized in humans[6] in food-induced anaphylaxis. In contrast, IgE-independent mechanisms (including those mediated by IgG, by the complement system, and direct activation of mast cells and basophils by drugs) have been studied in experimental animal models[7–10] and barely in humans.[11,12]

The diagnosis is carried out at the time of the reaction and is always based on clinical symptoms. Therefore, it often depends on the qualifications and experience of the specialist that assesses the patient since objective biomarkers that fully discriminate an anaphylactic event do not exist. Although, measurement of serum tryptase[13] and histamine[14] at the time of the reaction may support the diagnosis, the level of these biomarkers is not altered in all cases nor it is correlated with the course of the reaction, the severity and the cause or allergen trigger. Moreover, diagnosis by both biomarkers presents sensitivity and specificity limitations [15]. Thus, their usefulness is currently questioned.[16] The treatment of anaphylaxis targets only symptom control as no specific treatment exists. Injectable epinephrine is universally agreed as the first-line therapy.[17–19]

Key Messages

- Different triggers in humans (Food/Drug) have different metabolic profiles related to their action's mechanism.
- Severity of anaphylactic reaction produces different metabolic changes with a higher number in moderate.
- At basal state, different metabolic changes were found between moderate and severe anaphylactic reactions.

Metabolomics is the science used to characterize the metabolic response in a pathology.[20] Because of this, it is a promising tool in the study of anaphylactic reactions. The metabolome is closely linked to the phenotype and provides an extremely useful tool in the characterization of the disease. Although there is no single technique that detects the entire metabolome, the combination of mass spectrometry (MS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) provides complementary information that allows to identify a wider range of metabolites per sample.[21] Compared to other omics, such as transcriptomics or genomics, suitable validation of metabolites found after exploratory studies is carried out through the development of analytical methodologies. This process is usually laborious depending on the number of metabolites and their



GRAPHICAL ABSTRACT

For the first time, the metabolic profile of anaphylaxis in a real-life set-up was characterized and provide evidence that different triggers (Food and Drug) and severity (Moderate and Severe) induce differential metabolic changes improving clinical practice. Eighteen episodes of anaphylaxis with a full clinical characterization were analyzed by metabolomics. The time points were: acute phase (T1), recovery phase (T2) and basal state (T0). Basal status might identify high risk patients, thus opening new ways to understand, diagnose and treat anaphylaxis

physicochemical properties and is conditioned by the availability of commercial standards. Furthermore, this validation should be performed in bigger cohorts. Studies using metabolomic fingerprinting with a reduced number of samples have succeed in other allergic phenotypes such as asthma and food allergy.[20,22–24]

Anaphylaxis is a field yet to be explored since there are still no biomarkers that allow a sensitive and specific molecular diagnosis nor the severity of the reaction.[25] There are no indicators that predict the risk of certain patients suffering a severe allergic reaction. In many cases, the pathway by which the reaction takes place, dependent or independent of IgE, is also uncertain. Despite its great potential, metabolomics has been applied very slightly in human anaphylaxis. The unpredictable occurrence and outcome promote relevant technical and ethical difficulties. Moreover, controlled provocation set-ups do not reflect severe life-threatening reactions since on behalf of patient safety challenges are stopped.[26–28]

The aim of this exploratory study was to characterize the metabolic changes in patients suffering from anaphylaxis triggered either by foods or drugs in a real-life set-up comparing their acute phase (T1) versus their recovery phase (T2). Complementary, metabolite differences between moderate and severe anaphylactic reactions during the acute phase (T1) and basal state (T0) months later of anaphylactic reaction were obtained.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment

A prospective clinical and observational study of patients with anaphylactic reactions was performed. Patients of all ages and both sexes were recruited at outpatient clinics and the departments of

Emergency and other services at Hospital La Fe. All fulfilled clinical criteria of anaphylaxis, and severity was graded following the classification by Brown, et al.[29] Patients were classified as food, drug or idiopathic origin, as well as in mild, moderate or severe according to the number of organs affected and clinical symptoms. The allergy evaluation was conducted by the Allergology Service of Hospital La Fe. The ethical committee approved the study protocol, and all subjects were informed and provided written consent.

2.2 | Experimental design

Serum samples were taken during the acute moment of the reaction at the first moment of medical attention (<2 h, hereafter referred as 'T1') and after clinical recovery (approximately 2-4 h later, referred to as 'T2'). Patients were treated according to the Galaxy 2016 practical guide, using all necessary drugs to rescue them. Subsequently, between 2 and 3 months after the anaphylaxis, a sample was taken when the allergy evaluation was performed (basal state, called 'T0'). For sample collection at T0, patients were asked to have a fasting time of 8 h and to avoid regular medication. Medication received before sample collection at T1, T2 and T0 have been included in a table (see Table S1). A scheme of the experimental design is shown in Figure 1.

2.3 | Sample Collection and clinical parameters

Samples were collected in a vacutainer tube (Ref. 368965) and processed immediately after blood extraction following specific standard operating procedures.[30–32] Full details are described at Supporting Information (SI-Part 1). Sample aliquots were stored at −80°C until further analyses.

2.4 | Tryptase determination

Serum tryptase was measured following the manufacturer's instructions using the UniCAP-Tryptase fluoro-enzyme-immunoassay (FEIA) (Thermo Fisher Scientific, Uppsala, Sweden). Tryptase elevation was considered when a 20% (±2 ng/ml) of increment from baseline was observed.

2.5 | Metabolomic analyses

Samples were measured using a multiplatform analysis: ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS, Agilent 6550) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR, Bruker 500MHz). UPLC-MS and ^{1}H-NMR analyses were carried out in the Analytical Unit and in the Drug Discovery Unit, respectively, both at the Health Research Institute La Fe. Full descriptions following previous publications are available in SI-Part2[30–32]. Regarding UPLC-MS, a tentative identification was performed using an online software called CEU Mass Mediator tool.[33,34] Tandem mass spectrometry was used to confirm the annotation. Data were uploaded to Metabolomics Workbench webpage (ST001655 and ST001656).

3 | RESULTS

3.1 | Patient characteristics and clinical outcome

A total of 18 anaphylactic episodes occurring in 12 women (67%) and 6 men (33%) with a mean age of 42 years old (±20.1 SD) were analysed (Table 1). After the allergological evaluation, the episodes of anaphylaxis were classified according to the identified (or suspected) trigger. The resulting groups were 9 drug allergy (50%), 6 food allergy

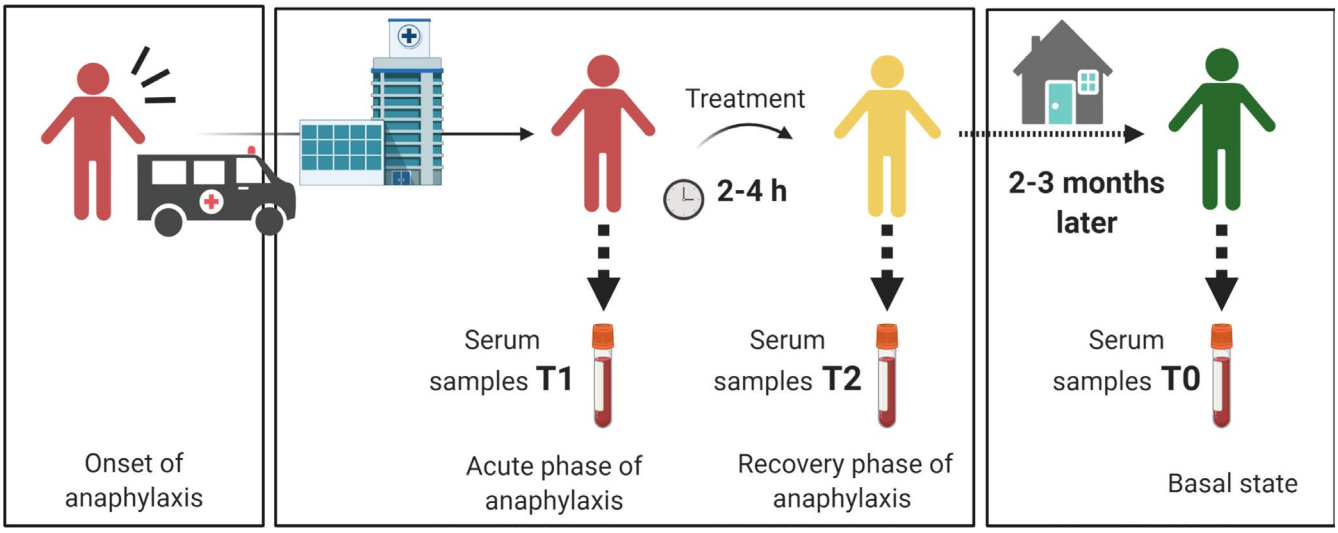


FIGURE 1 Experimental design. T, time-point

TABLE 1 Clinical, allergy and therapeutic characteristics of patients with anaphylactic reactions

Anaphylaxis trigger	Code	Demographics		Systems involved					Sev	Tryptase levels			Diagnostic allergy[&]	Allergy study			
		Age (y)	Gender	Skin	Brea.	Diges.	Neur.	CV		Time	Value	Incr.		SPT	BAT	sIgE	STC
FOOD	P2	10	W	YES	YES	NO	NO	NO	MOD	T0	1.7	YES	Walnut	POS	ND	POS	ND
										T1	3.8						
										T2	2.5						
FOOD	P8	31	W	YES	YES	NO	NO	NO	MOD	T0	3.5	NO	Mussel	POS	ND	POS	ND
										T1	5.4						
										T2	4.9						
FOOD	P9	27	W	YES	YES	NO	NO	NO	MOD	T1	4.1	NO	Cashew	POS	ND	POS	ND
										T2	4.4						
FOOD	P10	52	W	YES	YES	NO	NO	NO	SEV	T0	5.7	YES	LTP allergy (fruit)	POS	ND	POS	ND
										T1	9.1						
										T2	5.1						
FOOD	P15	21	W	YES	YES	NO	NO	NO	MOD	T0	1.8	YES	LTP allergy (salad)	POS	ND	POS	ND
										T1	11.2						
										T2	13.0						
FOOD	P17	16	M	YES	YES	NO	YES	NO	MOD	T0	1.0	YES	LTP allergy (nut)	POS	ND	POS	ND
										T1	8.3						
										T2	9.5						
DRUG	P1	66	M	YES	YES	NO	NO	NO	MOD	T0	8.3	YES	Carmellose	POS	POS	NA	ND
										T1	10.1						
										T2	19.6						
DRUG	P3	33	M	YES	YES	NO	NO	NO	MOD	T0	4.6	NO	Iomeprol	NEG	POS	NA	ND
										T1	4.6						
DRUG	P4	68	M	YES	YES	NO	YES	YES	SEV	T0	6.4	YES	Amoxicillin	POS	NEG	POS	ND
										T1	20.3						
										T2	24.0						
DRUG	P7	54	W	YES	YES	YES	YES	NO	SEV	T0	6.3	YES	Moxifloxacin	POS	NEG	NA	ND
										T1	17.8						
										T2	23.5						
DRUG	P12	39	W	YES	NO	NO	YES	YES	SEV	T0	1.5	YES	Metamizole	POS	POS	NA	ND
										T2	14.6						

(Continues)

TABLE 1 (Continued)

Anaphylaxis trigger	Code	Demographics		Systems involved					Sev	Tryptase levels			Diagnostic allergy[&]	Allergy study			
		Age (y)	Gender	Skin	Brea.	Diges.	Neur.	CV		Time	Value	Incr.		SPT	BAT	sIgE	STC
DRUG	P13	65	W	YES	YES	NO	NO	YES	SEV	T0	5.0	YES	Diclofenac	POS	NEG	NA	POS
										T1	9.2						
										T2	14.0						
DRUG	P14	79	M	YES	YES	NO	NO	NO	MILD	T0	10.2	NO	Ampicillin	POS	ND	POS	ND
										T1	11.4						
DRUG	P16	37	W	YES	NO	NO	NO	NO	MOD	T0	5.0	YES	Clavulanic acid	POS	ND	NA	ND
										T1	86.7						
										T2	76.7						
DRUG	P18	50	W	YES	NO	NO	YES	YES	SEV	T0	5.9	YES	Celebrex	NEG	ND	ND	POS
										T1	14.0						
										T2	9.3						
IDIOPATHIC	P5	60	M	YES	YES	NO	NO	NO	MOD	T1	4.9	NO	Idiopathic	NEG	ND	NEG	NEG
										T2	3.2						
IDIOPATHIC	P6	36	W	YES	YES	NO	ND	YES	SEV	T0	3.0	NO	Idiopathic	NEG	ND	NEG	NEG
										T1	3.8						
										T2	3.1						
IDIOPATHIC	P11	21	W	YES	YES	NO	YES	YES	SEV	T0	4.1	NO	Idiopathic	NEG	NEG	ND	NEG
										T1	4.6						
										T2	4.7						

Abbreviations: BAT, basophil activation test; Brea., breathing; CV, cardiovascular; Diges., Digestive; Incr., increase; M, man; MOD, moderate; MT0, basal time (2–3 months after reaction);NA, not available; ND, not performed; NEG, negative; Neur., neurologic; POS, positive; Sev, reaction severity; SEV, severe; SPT, skin prick test; STC, specific test challenge; T1, acute phase of reaction; T2, recovery phase; W, woman; y, years old.

[&]For diclofenac and metamizole, LTP-mediated anaphylaxis was rejected and the reaction was purely due to the NSAID.

(33%) and 3 idiopathic (17%). Regarding the severity of the anaphylactic reaction, 9 were moderate (50%), 8 severe (44%) and 1 mild (6%). The results showed an elevation of tryptase during anaphylaxis in 66% of patients with food reactions (4 out of 6) and 88% in those with drug reactions (7 out of 8). Interestingly, patients with reactions classified as idiopathic did not show elevation of tryptase during the reaction. These three patients were excluded from the following analyses since their cause of anaphylaxis could mislead the analysis. All patients recovered favourably from the reaction after treatment and were stabilized within a few hours (2–4 h). Intramuscular epinephrine was used as first-choice treatment for the anaphylactic reactions in 8 patients (45%). All patients were treated with antihistamines and corticosteroids (Table S1).

Drug and food triggers were the most common causes of anaphylaxis in this study, and we will focus on these groups (Table 2). Inside the food group, most patients presented moderate reactions except one severe case. In the drug group, five patients had severe reactions, three were moderate and one mild. The groups were balanced in terms of sex, severity or allergen trigger. The age was significantly higher in drug and severe groups compared to food and moderate groups, respectively ($p \leq 0.004$). Furthermore, the number of leucocytes was found to be significantly higher in food anaphylaxis compared to the drug anaphylaxis group ($p = 0.03$). The rest of the clinical parameters did not show significant differences between the groups of study.

3.2 | Metabolic profiling

Metabolomic profile of samples was obtained using LC-MS (positive and negative) and ^{1}H-NMR. Quality of LC-MS data was assessed by the clustering of quality control injections in PCA (Figure S1). Samples were explored following two different approaches: (1) based on the progression of the anaphylaxis reaction: comparing T1 *vs* T2 in the different anaphylactic conditions (food, drug, food + drug, severe and moderate anaphylaxis), and (2) based on the effect of the severity of the reaction in a particular time-point (severe *vs* moderate either at T0 or T1).

3.3 | Food anaphylactic reactions present a specific metabolic fingerprint

To test whether the drug- and food-triggered anaphylaxis cases experienced the same metabolic changes between T1 and T2, both groups were compared together ('food + drug T1 *vs* T2') and separately ('food T1 *vs* T2' and 'drug T1 vs T2') using multivariate statistics. We observed that only food anaphylaxis models 'T1 *vs* T2' in LC-MS positive mode showed good quality parameters for PCA and OPLSDA (Figure 2A,B). In particular, the cross-validated OPLSDA model (R^2 = 1.00 and a Q^2 = 0.79) showed a complete separation of the groups (Figure 2B). On the other hand, no model was obtained for drug or food + drug anaphylaxis, which means no major differences in 'T1 *vs* T2'.

The 'food anaphylaxis T1 *vs* T2' model was thought to reflect metabolic changes specifically associated with IgE-mediated anaphylactic reaction. To test this hypothesis, we used the ability of the model to classify the drug anaphylaxis samples. Figure 2C shows the prediction plot for the drug samples ($n = 9$). Interestingly, the prediction model separated the drug anaphylaxis samples into two groups. On one hand, 4 patients, allergic to antibiotics (beta-lactam: ampicillin, amoxicillin and clavulanic acid, and quinolone: moxifloxacin), were perfectly classified based on the time from anaphylactic reaction (T1 or T2) with a prediction score >75%. On the other hand, 5 patients, from which 3 suffered anaphylactic reactions caused by nonsteroidal anti-inflammatory drugs (NSAIDs: celecoxib, metamizole and diclofenac), 1 patient allergic to carmellose, 1 patient allergic to a contrast medium (iomeprol), were not classified in any food anaphylaxis time (T1 or T2; Table S2) with a prediction score <75%.

Focussing now in the 'food anaphylaxis T1 *vs* T2' in LC-MS positive, 73 significantly altered metabolites were obtained by univariate analysis. These were represented using a heat map with a hierarchical clustering (Figure 2D), and all patients were correctly grouped. Identification analysis allowed to observe an alteration in the levels of phospholipid-related metabolites, including phosphatidylcholines such as PC (16:0/16:1), lysophosphatidylethanolamine (LysoPE(19:0)/LysoPE(P-19:1)) and choline (Figure 2E). Furthermore, based on the results of the ^{1}H-NMR analysis of 'food T1 *vs* T2', significantly increased levels of acetate, phenylalanine, lysine, creatine and glutamine were characteristic of samples in T1 (Table S3).

3.4 | Severity of the reaction during time (T1 vs T2)

Once we established the differences regarding the trigger, we analysed the metabolic changes associated with the severity. Therefore, 'T1 *vs* T2' were compared independently in moderate and severe anaphylaxis reactions.

The most relevant model was obtained for 'moderate T1 *vs* T2' using the ^{1}H-NMR data. Separation of groups was observed both in PCA and OPLSDA models (Figure 3A,B). The cross-validated OPLSDA model showed $R^2 = 0.78$, $Q^2 = 0.56$, which correctly classified 93% of the samples from the model (13 out of 14 patients). Moreover, a heat map was built using the ^{1}H-NMR data that showed statistically significant differences between 'moderate T1 *vs* T2' ($n = 111$; $p < 0.05$), illustrating a clear metabolic signature (Figure 3C). Moderate reaction group was characterized by the increase in T1 of lactate, acetate, arginine, glutamine, isoleucine, leucine, valine, phenylalanine, proline and creatinine among others (Table S4). The trajectories of these metabolites are represented in Figure 3D. On the other hand, in LC-MS, choline was found increased at T1, while PCs and PEs were decreased.

All these changes were not observed in the 'severe T1 *vs* T2' comparison. However, the univariate analysis in LC-MS revealed an increase in PC(20:4/20:4) and a PE metabolite at T1 (Table S5).

TABLE 2 Patients and summary of clinical variables included in each comparison: (A) anaphylaxis trigger and (B) severity of the reaction

	(A) Anaphylaxis trigger			(B) Severity				
	Food	**Drug**	***p*-value**	**Mild**	**Moderate**	**Severe**	***p*-value**	**Test**
N° of subjects ($n = 15$)	6	9	—	1	8	6	—	
Mean age (SD) (years)	26 (14)	55 (16)	**0.004****	79 (0)[c]	30 (17)	55 (11)	**0.006****	*t* test
Female sex	5 (83%)	5 (56%)	0.580[b]	0%	63%	83%	0.245[a]	a: Chi-square (χ^2) b: Fisher's exact test
Anaphylaxis severity								Chi-square (χ^2)
Mild	0	1	0.156[a]	—	—	—	—	
Moderate	5	3		—	—	—		
Severe	1	5		—	—	—		
Anaphylaxis trigger	—	—						Chi-square (χ^2)
Food	—	—	—	0	5	1	0.156[a]	
Drug	—	—		1	3	5		
Measurement at T0								
N° of samples ($n = 11$)	4	7	—	1	6	4	—	Mann-Whitney *U* test
Total IgE (SD) (kUA/L)	367 (398)	891 (1819)	0.78	—	306 (239)	217 (323)	0.47	
Clinical parameters at T1								
N° of samples ($n = 8$)	5	3	—	—	5	3	—	Mann-Whitney *U* test
Creatinine (SD) (mg/dl)	0.883 (0.121)	0.796 (0.223)	0.571	—	0.828 (0.151)	0.830 (0.275)	1.000	
Leucocytes (SD) (/μL)	12826 (2975)	8164 (1778)	**0.036***	—	9580 (2064)	10466 (5127)	1.000	
Lymphocytes (SD) (/μL)	3116 (1932)	3932 (1167)	0.393	—	3858 (1778)	3240 (632)	0.786	
Eosinophils (SD) (/μL)	90 (51)	136 (149)	0.928	—	158 (139)	53 (5)	0.074	
Platelets (SD) (/μL)	301666 (42524)	281600 (54173)	0.571	—	293400 (49847)	282000 (54442)	0.785	
Prothrombin time (SD) (s)	ND	ND	—	—	14.3 (1.767)[d]	12.9 (1.501)	0.571	
Reactive protein (SD) (mg/L)	ND	ND	—	—	2.133 (0.751)[e]	2.067 (1.498)	1.000	

Student's *t* test, Mann-Whitney, chi-square (χ^2) or Fisher's exact test were applied depending on the distribution and type of the data using SPSS (v. 24.0); *$p < 0.05$; **$p < 0.01$, ND: no enough data for statistics, [c]; not included in the statistics, [d]; $n = 4$, [e]; $n = 3$. Idiopathic anaphylaxis and other causes are excluded.

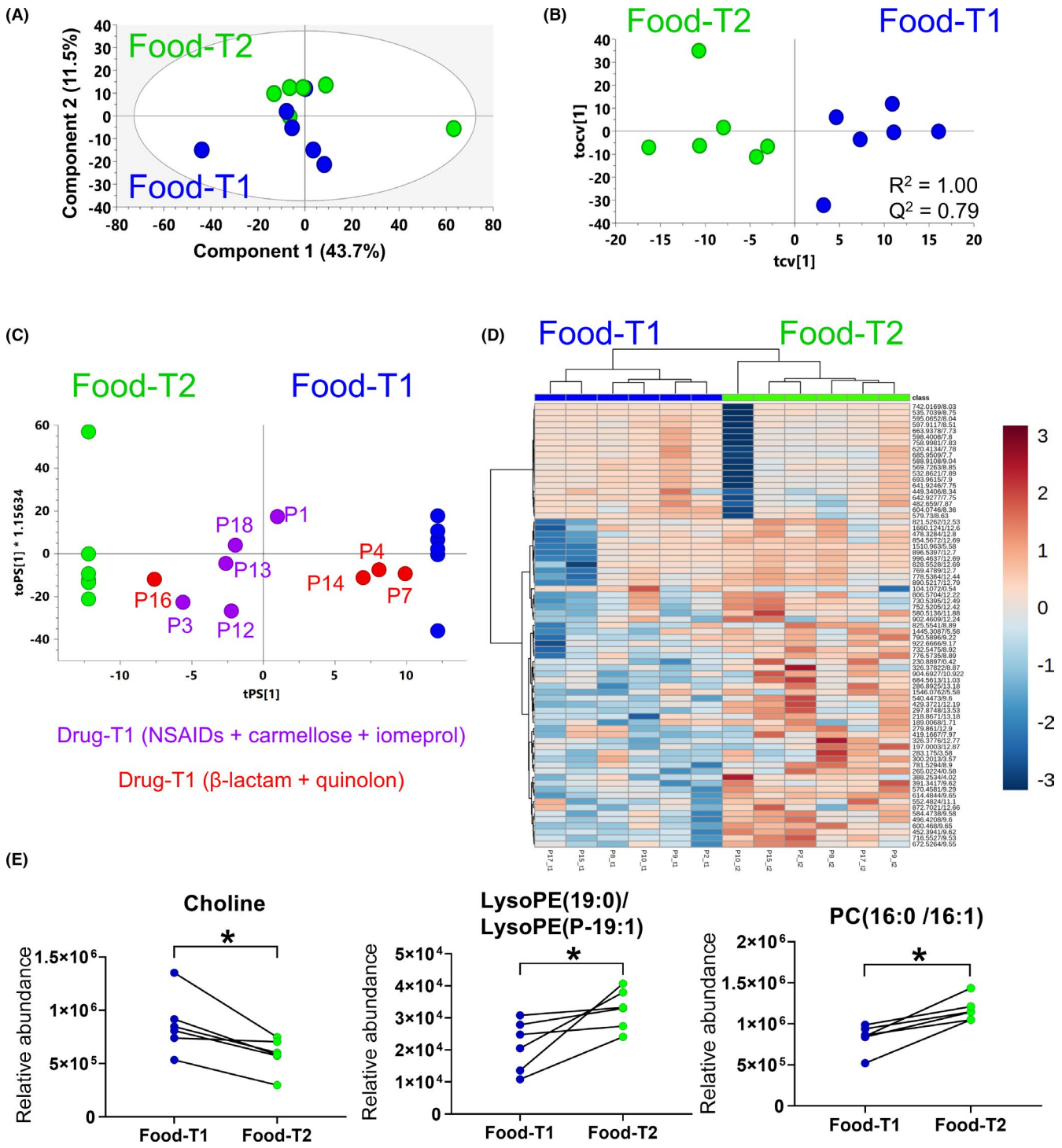


FIGURE 2 Progression findings from patients with food anaphylaxis from T1 at the acute phase to T2 at recovery using LC-MS in positive mode. (A) PCA and (B) OPLSDA cross-validated models for food anaphylaxis between T1 and T2; $n = 6$; log transformation and centre scaling was used for the models. (C) Prediction model using the samples of food anaphylaxis at T1 (blue circle) and T2 (green circle) to predict patients allergic to drugs at T1. Patients allergic to antibiotics are depicted in red circles, whereas allergic patients to NSAIDs drugs, iomeprol and carmellosa were painted in purple. (D) Heat map using hierarchical clustering of the samples of food anaphylaxis at T1 and T2 (represented in columns) and features (in rows) using signals showing statistically significant differences ($n = 73$) from LC-MS in positive mode. Red cells represent higher levels of the specific metabolite in that sample, whereas blue cells represent lower levels. Samples and metabolites are clustered according to their similarity. Wilcoxon signed-rank test with a Benjamini-Hochberg correction was used to detect statistical significance ($p < 0.05$). Metabolites are defined as mass (Da)/retention time (min). (E) Trajectories of significant identified metabolites in food anaphylaxis group between T1 and T2; $^*p < 0.05$

3.5 | Metabolic differences due to severity at different time-points T1 and T0

Finally, metabolic differences due to severity during the anaphylactic episode (at T1) and at the basal point (at T0) were analysed independently.

Using univariate analysis, we observed that at the moment of the anaphylactic reaction (T1), 7 metabolites from both techniques (LC-MS and ^{1}H-NMR) were significantly higher in the severe group compared to the moderate group. These metabolites included cortisol, glucose, lipids (-CH=CH-CH2-CH=CH-), lipids (-CH=CH-) and lipoprotein methyl group signal (Table S6).

Likewise, at the basal time-point (T0), the levels of 10 metabolites using both techniques were significantly higher in the severe group compared to the moderate group such as oleamide, PC(14:0/20:4), lactate, lipids (-CH2-CH=CH), lipids (-CH=CH-CH2-CH=CH-) and lipids (-CH=CH-). Meanwhile, betaine and cortisol were lower (Table S7). Trajectories of these metabolites showed that most of them are increased in severe group regardless of the time-point (T1 or T0, Figure 4).

4 | DISCUSSION

The increasing prevalence of anaphylaxis reactions points out to an urgent need for progress in the search of biomarkers for early diagnosis and accurate therapy. Studies of anaphylaxis in humans are still one of the greatest challenges in allergy research, and to date, they are very limited due to the clear difficulties they entail.

In this exploratory study, most anaphylactic cases occurred in women. They were mostly attributed to drugs, being graded as the most serious followed by foods. Drug mediated reactions were more common in elderly patients. All these observations match with previous studies of anaphylaxis.[35] Regarding the clinical parameters, tryptase was not elevated in all the cases (88% in drug and 66% in food anaphylaxis) as has been previously reported.[15] Interestingly, patients with reactions classified as idiopathic did not show elevation of tryptase during the reaction, explaining the heterogeneity of the trigger mechanism. Other clinical parameter was the number of leucocytes, which was significantly increased in food compared to drug anaphylaxis. This fact also has already been described by other authors.[36] Further studies are needed to clarify this result.

Regarding food anaphylaxis, we observed a clear metabolic pattern during the time of the anaphylactic reaction ('T1 *vs* T2'), which was not observed in drug or food + drug anaphylaxis. This finding could be related to different mechanisms of action triggered by different allergens. Food anaphylaxis reactions, which are mainly IgE-mediated, were confirmed by sIgE measures, whereas the mechanisms of drug-triggered reactions are described to be more heterogeneous.[37] This fact was confirmed with the prediction of the well-characterized drug anaphylaxis of β-lactam and NSAIDs. β-lactam antibiotic-triggered reactions, which are known to be IgE-mediated, were clustered together within one of the groups of food anaphylaxis model (T1 and T2).

Other IgE-independent reactions like those caused by NSAIDs were not clustered within the food model. However, the mechanisms of other drug-triggers like iomeprol, carmellose and moxifloxacin are not clearly defined.[38–43] Iomeprol (not classified with food anaphylaxis with a prediction score of 73%) has been strongly suggested to trigger IgE-mediated hypersensitivity by a diagnostic workup using basophil activation test.[39,40] Carmellose (not classified with food anaphylaxis with a prediction score of 54%) was reported having an unclear mechanism.[38] Finally, moxifloxacin (classified with food anaphylaxis prediction score of 91%) follows a mixed IgE and MRGPRX2-mediated mechanisms.[41–43]

The metabolic changes observed in food anaphylaxis at 'T1 *vs* T2' were that phospholipids significantly decreased, while choline, a substrate of PCs, was found increased, all at T1. These were mainly PCs, from which palmitic acid (C16:0), which displays anti-inflammatory activities, and palmitoleic acid (C16:1), that enhances the inflammatory reactions, were the most frequent fatty acids chains contained in their structure.[44–47] These fatty acids are freed from PCs by the action of the phospholipase A2 enzyme (PLA2), which promotes mast cells maturation.[48] This suggests that these PCs are key regulators in the acute phase.

To complement this, we found increased levels during the acute reaction (T1) of (1) acetate, which is released in catabolic and metabolic stress conditions.[49] (2) Glutamine, which is essential during catabolic situations, as the rate of glutamine consumption by all immune cells is similar or greater than glucose.[50] Moreover, glutamine promotes enterocyte proliferation, regulates tight junction proteins, suppresses proinflammatory signalling pathways and confers protection against apoptosis and cellular stresses during normal and pathological conditions.[51] It is known that during anaphylaxis, mast cells, basophils and neutrophils induce hypotension, vascular hyper-permeability and an endothelial production of nitric oxide (NO).[52] (3) Phenylalanine can be involved in NO production, reducing superoxide species and enhancing vascular function.[53] (4) Histidine is the precursor of histamine which is synthesized by the action of the histidine decarboxylase enzyme inside mast cells and basophils.[54] As histamine is released in T1 when the patient is recovered at T2, histidine would decrease since it could be used to replenish histamine in the mast cells granules.

To sum up, all these metabolic changes seem to be a reflection of anaphylaxis and suggest a rapid metabolic response to a series of defined innate defensive mechanisms and the elevated catabolism triggered in the first moments of the anaphylactic episode.

The moderate anaphylactic reactions were characterized by a decrease in several PCs at T1. The most frequent fatty acyl chains of these PCs were palmitoleic acid (C16:1), docosahexaenoic acid (C22:6; DHA), precursors of anti-inflammatory specialized pro-resolving mediators and arachidonic acid (C20:4; AA), related to inflammatory processes.[44,55] Furthermore, we detected increased levels of branched-chain amino acids (BCAA: leucine, isoleucine and valine) at T1. Elevated BCAA levels generate inflammation and oxidative stress in endothelial cells via mTORC1 pathway, thereby facilitating inflammatory cells adhesion and endothelial dysfunction.[56] Another metabolite which was increased at T1 was arginine.

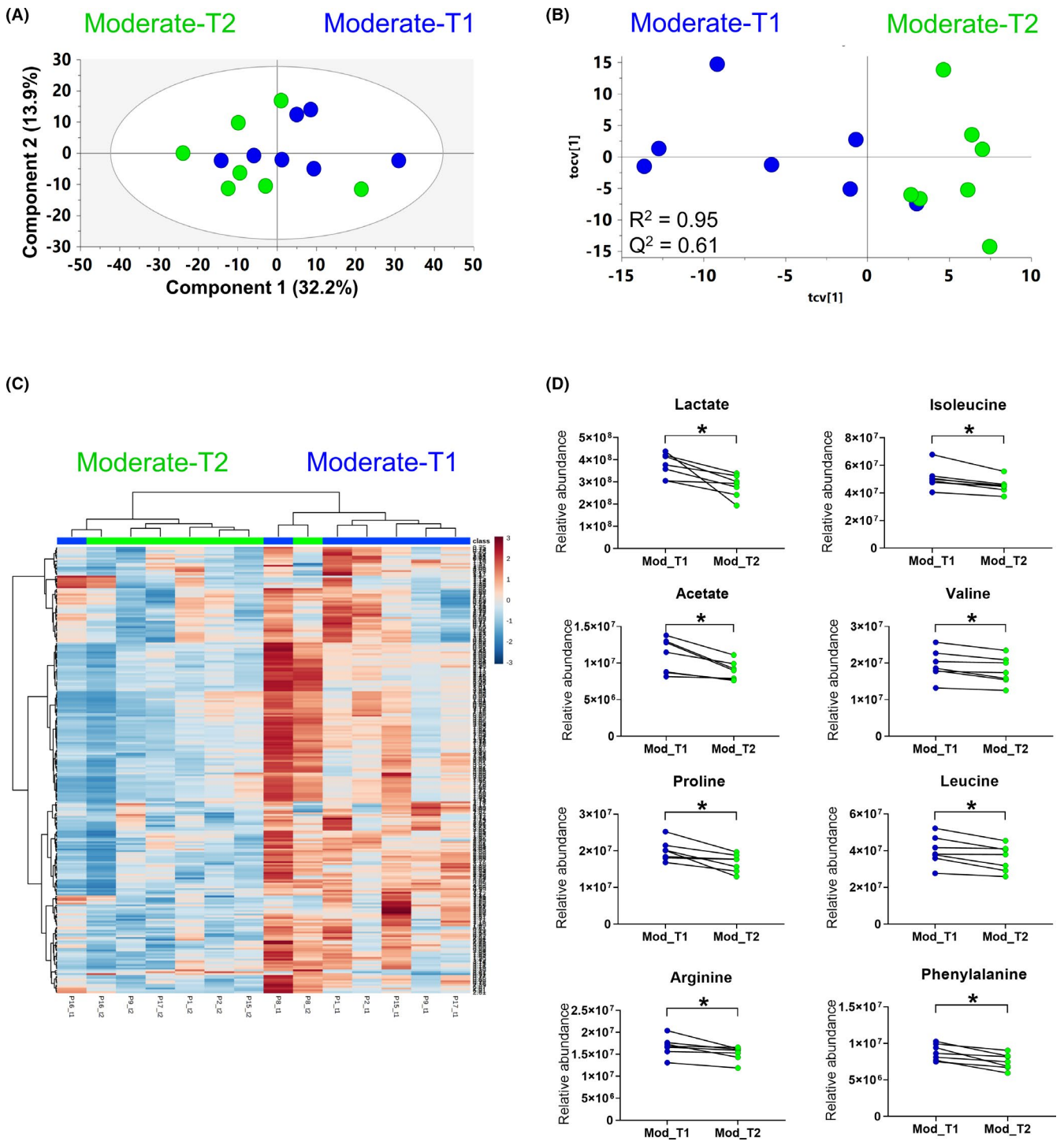


FIGURE 3 Progression findings from patients with moderate anaphylaxis from T1 at the acute phase to T2 at recovery using the ^{1}H-NMR data. (A) PCA and (B) OPLSDA cross-validated models for moderate anaphylaxis between T1 and T2; $n = 8$; Pareto scaling was used for the models using the ^{1}H-NMR data. (C) Heat map using hierarchical clustering of the samples of moderate anaphylaxis at T1 and T2 (represented in columns) and buckets (in rows) including significant buckets ($n = 111$) from ^{1}H-NMR analysis. Red cells represent higher levels of the specific NMR spectra region in that sample, whereas blue cells represent lower levels. Samples and features are clustered according to their similarity. Wilcoxon signed-rank test with a Benjamini-Hochberg correction was used to detect statistical significance ($p < 0.05$). (D) Trajectories of significant identified metabolites in moderate anaphylaxis group between T1 and T2; $^{*}p < 0.05$

During anaphylaxis, histamine binds to H_1 receptors. This stimulates endothelial cells to convert the amino acid arginine into NO.[4] These observed results point to an alteration of the energy metabolism. In particular, an increase in lactate, acetate and creatinine was detected at T1. High amounts of lactate, the resulting product of anaerobic glycolysis, are produced by innate immune cells during inflammatory activation.[57] Additionally, an increase in proline at T1 was observed. Recent findings suggest that proline is a stress

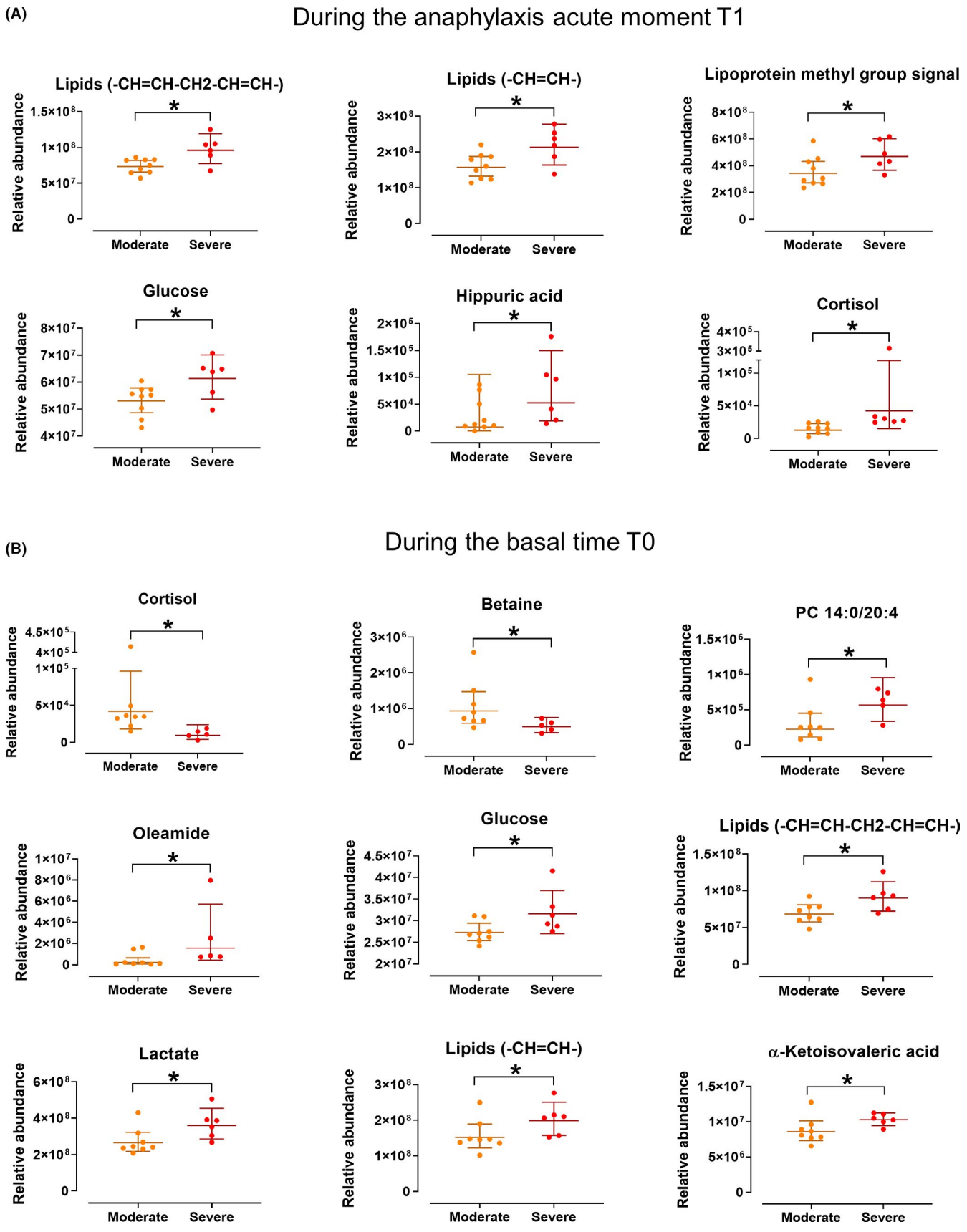


FIGURE 4 Trajectories of significant identified metabolites between moderate and severe groups at specific time-points: (A) at T1 in the acute phase and (B) at T0 in the basal point using the data from all techniques. Mann-Whitney *U* test with a Benjamini-Hochberg correction was used to detect statistical significance ($p < 0.05$)

substrate in inflammation, as it is used to generate superoxide radicals which initiate apoptosis, as well as an energy source.[58] Finally, increases in glutamine, phenylalanine and choline were also observed at T1 and have already been described for the model of food anaphylaxis 'T1 *vs* T2'.

To sum up, moderate anaphylactic reactions appear to be the result of proinflammatory metabolites, and some of them could be involved in the endothelium and immune cell participation.

On the other hand, for severe anaphylactic reactions between 'T1 *vs* T2', the AA fatty acyl chain of the PC could point to a more sustained inflammation by the severe individuals. This might be associated with a higher difficulty of the inflammatory system to recover and/or the heterogeneity of the patients regarding their recovery at T2.

If there are still many questions regarding the underlying mechanisms of action of anaphylaxis, the factors that determine their severity present an equal or even greater challenge. We analysed the metabolic differences between moderate and severe groups in the acute phase of the reaction (T1). The severe group presented higher levels compared to the moderate group of (1) glucose, which supports the enhanced cellular metabolism of the immune system, (2) lipids and lipoproteins, suggest a higher cell signalling and energy consumption and (3) cortisol—an endogenous metabolite with strong anti-inflammatory properties—which points to the mobilization of glucose reserves for energy and inflammatory modulation.

Analysis of samples at basal time (T0) was carried out to investigate risk factors for severe anaphylaxis. Severe reactions showed higher levels of glucose, lipids and oleamide, and lower amounts of cortisol and betaine compared to moderate reactions during the basal phase. Oleamide has been described to have anti-inflammatory and anti-allergenic functions in both *in vivo* and *in vitro* models.[59–61] The higher levels of oleamide detected in severe patients, months after the anaphylactic reaction, could suggest a protective metabolic state. Alternatively, betaine—an important tissue osmolyte—is *de novo* biosynthesized based on the choline oxidation, which is a precursor of PCs.[62,63] This would explain the low levels of betaine in patients who suffered severe reactions and who maintain a high level of serum phospholipids and therefore a lower use of choline to generate betaine.

The limitations of carrying out non-targeted metabolomic studies on anaphylaxis in a real-life set-up are unavoidable. As such, medication is present at one or another time before sample collection (T1 or T2). However, as not all the patients received the same emergency medication, the changes that were observed regarding the triggers or severity at T1 *vs* T2 can be truly associated with the disease. Another limitation is the lack of homogeneity among the cases by the trigger, age range and food intake which are factors that cannot be controlled in an emergency set-up. Complementary comparisons such as T1 *vs* T0 and food *vs* drug at T0 are of high interest and could be addressed in future studies following other experimental designs. Further validation in a bigger cohort will be necessary to confirm our results, avoiding any possible bias between study groups and increasing the power of the analysis.

The results of this exploratory study provide the first evidence that different anaphylactic triggers or severity induce differential metabolic changes along time or at specific time-point, respectively. These findings suggest to some extent, the immunological memory also extends to the metabolism of patients, especially those who experienced severe anaphylaxis. Despite the need for additional research to confirm these results, the metabolites identified in our exploratory study could shed light on the prediction of the risk of a severe anaphylaxis.

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CONFLICT OF INTEREST

Domingo Barber has received consultancy fees from ALK and Aimmune therapeutics companies. The rest of the authors have no conflict of interest. All authors have read and approved the manuscript.

AUTHOR CONTRIBUTION

DHFR was the PI and together with CP-C, AP-L and LP-C designed and supervised the research. MR, DO and LT performed the LC-MS based metabolomic analysis, data treatment and identification of metabolites. LP-C and AR-B performed the NMR metabolomic analysis. DO, LT, VE, CB, JJL, DB and AV contributed to the metabolomics interpretation. CP-C, JM-G, EI-E, RL-S and DHFR included all study patients. DB and AV supervised statistical analysis. All authors contributed to the writing of the manuscript and have given approval to the final version of the manuscript.

ETHICS APPROVAL

The ethical committee from 'Instituto de Investigación Sanitaria La Fe' approved the study protocol signed by the general director José Vicente Castell Ripoll and the board president Rafael Botella Estrada.

ORCID

David Obeso https://orcid.org/0000-0001-7875-7327
José Julio Laguna https://orcid.org/0000-0001-5909-8979
Domingo Barber https://orcid.org/0000-0002-5488-5700
Alma Villaseñor https://orcid.org/0000-0002-6652-2739

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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