Received: 24 November 2020 | Revised: 3 April 2021 | Accepted: 8 April 2021

DOI: 10.1111/pai.13518

ORIGINAL ARTICLE

WILEY

Increased miR-21-3p and miR-487b-3p serum levels during anaphylactic reaction in food allergic children

Emilio Nuñez-Borque[1] | Sergio Fernandez-Bravo[1] | Pablo Rodriguez Del Rio[2,3] | Ebrahim Mohammed Alwashali[4] | David Lopez-Dominguez[5] | Maria Dolores Gutierrez-Blazquez[4] | Jose Julio Laguna[6,3] | Jaime Tome-Amat[7,3] | Julio Gallego-Delgado[8,9] | Alicia Gomez-Lopez[10] | Diana Betancor[10] | Javier Cuesta-Herranz[1,3,10] | Maria Dolores Ibañez-Sandin[2,3] | Alberto Benito-Martin[11] | Vanesa Esteban[1,3,12]

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

[2]Allergy Department, Foundation for Biomedical Research, Niño Jesus University Children's Hospital, Madrid, Spain

[3]Instituto de Salud Carlos III, ARADyAL Network, Madrid, Spain

[4]CAI Genomics and Proteomics, Proteomic Unit, Faculty of Pharmacy, Complutense University of Madrid, Madrid, Spain

[5]Clinical Biostatistics Unit, Instituto de Investigación Puerta de Hierro (IDIPHIM), Puerta de Hierro Majadahonda University Hospital, Madrid, Spain

[6]Allergy Unit, Allergo-Anaesthesia Unit, Faculty of Medicine, Hospital Central de la Cruz Roja, Alfonso X El Sabio University, Madrid, Spain

[7]Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Universidad Politécnica de Madrid, Madrid, Spain

[8]Department of Biological Sciences, Lehman College, City University of New York, New York, NY, USA

[9]Program in Biology, The Graduate Center, The City University of New York, New York, NY, USA

[10]Hospital Fundación Jiménez Díaz, Madrid, Spain

[11]Instituto Biomédico de Nutrición y Salud., IBIONS, Spain, Spain

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

Correspondence
Vanesa Esteban, Department of Allergy and Immunology, IIS-Fundación Jiménez Díaz, Avda. Reyes Católicos 2, Madrid 28040, Spain.
Email: vesteban@fjd.es

Funding information
Spanish Council Ministry of Science and Innovation; Ramón y Cajal Program, Grant/Award Number: RyC-12880-2013; the Instituto de Salud Carlos III, Grant/Award Number: PI18/00348; FEDER Thematic Networks and Cooperative Research Centers; RETICS ARADyAL, Grant/Award Number: RD16/0006/003, RD16/0006/0013, RD16/0006/0026 and RD16/0006/0033; SEAIC, Grant/Award Number: 19_A08; Alfonso X el Sabio University Foundations; Community of

Abstract

Background: Anaphylaxis is the most severe manifestation of allergic disorders. The poor knowledge of its molecular mechanisms often leads to under-diagnosis. MicroRNAs (miRNA) regulate physiologic and pathologic processes, and they have been postulated as promising diagnostic markers. The main objectives of this study were to characterize the human miRNA profile during anaphylaxis and to assess their capacity as diagnostic markers and determine their participation in the molecular mechanisms of this event.

Methods: The miRNA serum profiles from the acute and baseline phase of 5 oral food-challenged anaphylactic children (<18 years old) were obtained by next-generation sequencing (NGS). From the panel of statistically significant miRNAs obtained, several candidates were selected and analyzed in 19 anaphylactic children by qPCR. We

Abbreviations: ECs, endothelial cells; FC, fold change; FDR, false discovery; HMDD, human miR disease database; HMVEC-D, human dermal microvascular endothelial cell; miR/miRNA, microRNA; mRNA, messenger RNA; NGS, next-generation sequencing; PAF, platelet-activating factor; PCA, principal component analysis; RISC, silencing complex induced by miRNA; SBA, systems biology analysis; Th, T helper lymphocyte.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2021 The Authors. *Pediatric Allergy and Immunology* published by European Academy of Allergy and Clinical Immunology and John Wiley & Sons Ltd.

Madrid; FOODAL, Grant/Award Number: CM_P2018/BAAA-4574

Editor: Hugh Sampson

performed system biology analysis (SBA) on their target genes to identify main functions and canonical pathways. A functional in vitro assay was carried out incubating endothelial cells (ECs) in anaphylactic conditions.

Results: The NGS identified 389 miRNAs among which 41 were significantly different between acute and baseline samples. The high levels of miR-21-3p (fold change = 2.28, *P* = .006) and miR-487b-3p (fold change = 1.04, *P* = .039) observed by NGS in acute serum samples were confirmed in a larger group of 19 patients. The SBA revealed molecular pathways related to the inflammation and immune system regulation. miR-21-3p increased intracellularly and in acute phase serum after EC stimulation.

Conclusions: These findings provide, for the first time, some insights into the anaphylactic miRNA serum profile in children and point to miR-21-3p and miR-487b-3p as candidate biomarkers. Furthermore, the SBA revealed a possible implication of these molecules in the underlying molecular mechanisms. Moreover, ECs increased miR-21-3p intracellularly and released it to the environment in response to anaphylaxis.

KEYWORDS

anaphylaxis, biomarker, endothelial cells, microRNA, next-generation sequencing, systems biology analysis

1 | INTRODUCTION

Anaphylaxis, the most severe allergic manifestation, is rapid in onset and life-threatening. Food, drugs, and Hymenoptera venoms are the most frequent triggers. Over the last decades, the rate of anaphylaxis has increased in all age groups.[1] Unlike other allergic diseases, it is a systemic reaction that can affect the skin and respiratory, gastrointestinal, neurologic, and cardiovascular systems. Mechanistically, sensitization occurs when an allergen stimulates the polarization of T helper lymphocytes (Th) to a Th_2 response. Subsequently, plasma cells secrete specific IgE that binds to the FcεRI surface receptors on mast cells and basophils, the effector cells of the reaction. When further contacts with the allergen take part, cross-linking of the high-affinity FcεR occurs inducing effector cell degranulation and the release of various mediators producing pathologic symptoms. However, this mechanism alone cannot explain all the reactions.[2,3]

Anaphylaxis affects several systems being the circulatory one of the most important due to its key role in the development of the anaphylactic shock. Among its components, the endothelium, conformed by endothelial cells (ECs), is a dynamic structure, which regulates plenty of physiologic processes.[4] Vasoactive and inflammatory mediators released by effector cells during anaphylaxis destabilize this barrier giving rise to part of the diverse pathologic manifestations of this event.[4,5]

Anaphylaxis is often misdiagnosed, mainly because of the lack of universally accepted diagnostic criteria and biomarkers.[3] Generally, it is carried out according to the clinical symptoms, which are common to many other pathologies, so the confirmation through in vitro markers is usually mandatory.[6,7] The availability of a sensible, specific, and easy detectable biomarker would support a more precise diagnostic marker.[6] Currently, the level of serum tryptase is the most relevant in vitro marker used in the clinical practice.[7,8] This molecule is released by mast cells and basophils, increased during anaphylaxis.[6-8] Unfortunately, it does not correlate with a large number of cases and clear-cut diagnosis thresholds have not been established.[6,8]

> **Key message**
>
> miR-21-3p and miR-487b-3p are candidate biomarkers in serum from anaphylactic children. Systems biology analysis reveals a possible implication of these molecules in different molecular mechanisms, and specifically, endothelial cells (ECs) increased miR-21-3p intracellularly and released it to the environment in response to anaphylaxis.

MicroRNAs (miRNAs) are small non-coding RNA molecules (~22 nucleotides) that regulate the translation of messenger RNAs (mRNAs). Their genes are mainly transcribed by RNA polymerase II and processed by Drosha and DICER, resulting in a mature duplex miRNA whose strands separate and generate the silencing complex induced by miRNA (RISC). This, through the "seed" sequence (nucleotides 2-8) of the 5' miRNA, will bind to the complementary region in the 3' UTR of its target mRNA degrading it or repressing its translation.[9] Therefore, miRNAs participate in the regulation of several cellular functions maintaining a physiologic balance. According to the databases miR2-Disease and human miR disease database (HMDD), both increased and decreased levels are implicated in a wide range

of pathologies.[10] Currently, the role of these molecules has been described taking part in the molecular mechanisms in diverse allergic diseases. Furthermore, miRNAs have been proposed as non-invasive biomarkers in several of them due to their stability in serum and easy obtaining.[11] However, no miRNAs have been described in human anaphylaxis and their molecular relevance is unknown.

The aim of this study was, for the first time, to assess the serum miRNA profile of anaphylaxis in children, and to evaluate the possible implication of these molecules in the underlying molecular bases of the reaction and their capacity as biomarkers.

2 | METHODS

An expanded Methods section is available in the Supplementary material.

2.1 | Patient's selection

Children undergoing open oral food challenges who were admitted at the Hospital Niño Jesus, Madrid, due to anaphylaxis were invited to participate before the challenge started. Anaphylaxis and severity were described according to the previously published references.[12-14] Serum from 19 children (<18 years old) was collected within the first 30 minutes from the start of the reaction (acute phase) and baseline determinations were performed 14 days later (basal phase). Considering the heterogeneity of the reactions, the baseline was used as a control of each acute sample. Serum tryptase levels were measured using UniCAP (Thermo Fisher Scientific).

2.2 | Circulating serum miRNA profile by next-generation sequencing

In order to determine the circulating serum miRNA profile of anaphylaxis, acute and baseline samples from the 5 children with the most severe clinical presentation were analyzed in the same batch by next-generation sequencing (NGS) at Qiagen Genomic Services.

2.3 | miRNA quantitation by qPCR

miRNAs were isolated from serum by the miRNeasy Serum/Plasma Advanced Kit and from ECs by MasterPure™ Complete DNA & RNA Purification Kit (Lucigen), retrotranscribed using the miRCURY LNA RT Kit, and quantified by qPCR with the miRCURY LNA SYBR Green PCR Kit (all from Qiagen) as their protocols indicate. Specific primers (Qiagen) were used to measure miRNAs of interest, and the $2^{-\Delta\Delta CT}$ method was applied to quantitate the different data[15] obtained in the LightCycler 96 Real-Time PCR System (Roche Life Science).

2.4 | Systems biology analysis

In silico SBA was performed using the Ingenuity Pathway Analysis software (Qiagen). The program provided different functional categories among which top disease and functions and canonical pathways were considered.

2.5 | In vitro serum/EC system

Human dermal microvascular ECs (HMVEC-D) were acquired from Lonza CC-2543 and maintained in EGM™-2MV BulletKit™ as previously described.[16,17] EC monolayers were incubated for 2 hours with: EGM-2 medium (negative control), a cocktail of anaphylaxis mediators, or serum samples (acute and basal) from 5 patients. Mediators included in the cocktail were histamine, platelet-activating factor (PAF), and thrombin (all from Sigma). miRNA determinations were performed in serum samples before and after EC contact and intracellularly at the end-point.

2.6 | Statistical analysis

Data obtained from NGS were analyzed with the statistical software R 3.5.3 (R Core Team, 2019). Normalization and relative quantitation were performed with the Prostar Pack (http://live.prostar-proteomics.org/) distributed by Bioconductor and implemented in R. The volcano plot was carried out also with the software R 3.5.3. However, the principal component analysis (PCA) and the heat map were realized in the website ClustVis (https://biit.cs.ut.ee/clustvis/).[18] Graphical representation and qPCR statistical evaluation were performed by using the software GraphPad Prism 6. Differences were considered significant at level of $P < .05$.

3 | RESULTS

3.1 | Patients' characteristics

The studied population includes samples from 19 anaphylactic patients from 4 to 17 years old (mean 11.3 ± 0.92), being 58% of them females. All reactions were triggered by food; specifically, 37% were induced by milk, 26% by egg, and 37% by other allergens. Referring to the severity and clinical classification, the anaphylactic reactions were classified as severe in 10% of the cases and moderate in 90%. However, none of them exceeded the reference threshold values of serum tryptase established by the manufacturer for the diagnosis of anaphylaxis.[7,19] A fulfilled description of the patients and their clinical characteristic is shown in Table 1.

3.2 | Characterization of circulating serum miRNA profile in anaphylactic patients

Anaphylaxis is a major health problem in the pediatric population, as both incidence and hospitalizations increase every year.[20]

TABLE 1 Clinical characteristics of anaphylactic patients included in this study

Patients				Symptoms						Signs		Severity	Tryptase	
Number	Age	Gender	Trigger	Skin	Mucous	Digestive	Respiratory	Nervous	Vascular	Hr	$SatO_2$	Grade	Acute	Basal
1	17	F	Milk	E; Pr		N; Ap				69	-	2	6.93	5.91
2	11	F	Egg			N; Ap; V; Dr	R			-	-	2	5.83	3.34
3	4	M	Cashew	E	Ag		R		T	136	98%	2	<1	<1
4	5	F	Roosterfish	E; Pr; U			W			83	98%	2	9.36	11.4
5	9	F	Milk			N; Ap; V; Dr	R			83	99%	2	5.5	<1
6	9	M	Egg	E; Pr		N; Ap; V; Dr	R		T	135	98%	2	5.21	3.01
7[a]	14	F	Egg	E; Pr	Ag	N; Ap	R	Dz	Hy	56	99%	3	2.6	2.29
8	11	F	Milk	U	Ag		R; W			89	97%	2	3.81	3.31
9[a]	15	M	Cheese	E			R; W			57	96%	2	8.03	5.69
10	13	F	Milk			Ap	R; W			77	98%	2	5.04	2.98
11[a]	5	M	Milk	E		Ap; V	Dy		Hy	85	94%	3	7.83	4.97
12	14	M	Egg	E	Ag	N; Ap; V; Dr	R			81	98%	2	2.88	2.44
13	10	F	Egg	E; Pr	Ag		W			117	-	2	3.89	3.78
14[a]	14	F	Nut	U	Ag		Cg	Dz		96	99%	2	6.63	5.65
15	7	M	Roosterfish		Ag		Cg			85	98%	2	3.84	2.53
16[a]	14	F	Milk	U		N; Ap	R; W			107	97%	2	8.74	1.72
17	11	F	Milk	E	Ag	N; Ap	R; Cg			100	99%	2	8.57	7.05
18	15	M	Peanut			Ap; V	Dy			93	99%	2	3.03	1.73
19	17	M	Hazelnut		Ag		Dy			90	100%	2	5.62	7.04

Note: Gender: M (male), F (female). Clinical symptoms—skin: E (erythema), U (urticaria), and Pr (pruritus); mucous: Ag (angioedema); digestive: N (nausea), V (vomit), Dr (diarrhea), and Ap (abdominal pain); respiratory: Dy (dyspnea), W (wheezing), R (rhinitis), and Cg (cough); vascular: Hy (hypotension) and T (tachycardia); and neurologic: Dz (dizziness). Clinical signs—Hr (heart rate) and $SatO_2$ (oxygen saturation). Tryptase values correspond to ng/mL.

[a]Patients used for the NGS.

Therefore, to characterize the circulating serum miRNA profile in anaphylactic patients, a pilot-scale study was performed with 5 children. Levels of serum miRNAs were determined by NGS, and a total of 389 were identified (Supplementary Table 1). A detailed description of the NGS quality controls is shown in Supplementary Figure 1. The PCA revealed similarity between samples belonging to biologic replicas and a separation between acute and basal groups (Figure 1A). Moreover, the dispersion of all identified miRNAs is shown in Figure 1B. After statistical analysis, data evidenced significant differences in the abundance of 41 miRNAs between both conditions (Supplementary Table 2; Figure 1C). Among these, 21 were enriched in the acute phase while 20 were decreased (Figure 1D). Through a comprehensive search in PubMed, it was observed that 37% of these miRNAs were previously described in the allergy field (Supplementary Table 3), but they have never been related to anaphylaxis.

3.3 | Serum miR-21-3p and miR-487b-3p are increased during the acute phase of anaphylaxis

Among the statistically significant miRNAs identified by NGS, several of them were chosen to further validate our results. Therefore, we extended the isolation, reverse transcription, and quantitation to 14 additional children's samples that were not previously texted in the NGS study. In total, 19 patients including 38 samples in both basal and acute conditions were analyzed. The results obtained showed that none of them exceeded the reference threshold values of hemolysis (≥7 cycles), presenting adequate conditions for further research (Supplementary Figure 2A). In addition, UniSp2, UniSp4, and UniSp5 were detected to be stepped between 5 and 7 cycles confirming the correct extraction of serum miRNAs (Supplementary Figure 2B). UniSp6 was amplified around cycle 18, demonstrating that the reverse transcription was also appropriately carried out (Supplementary Figure 2C). After confirming the quality

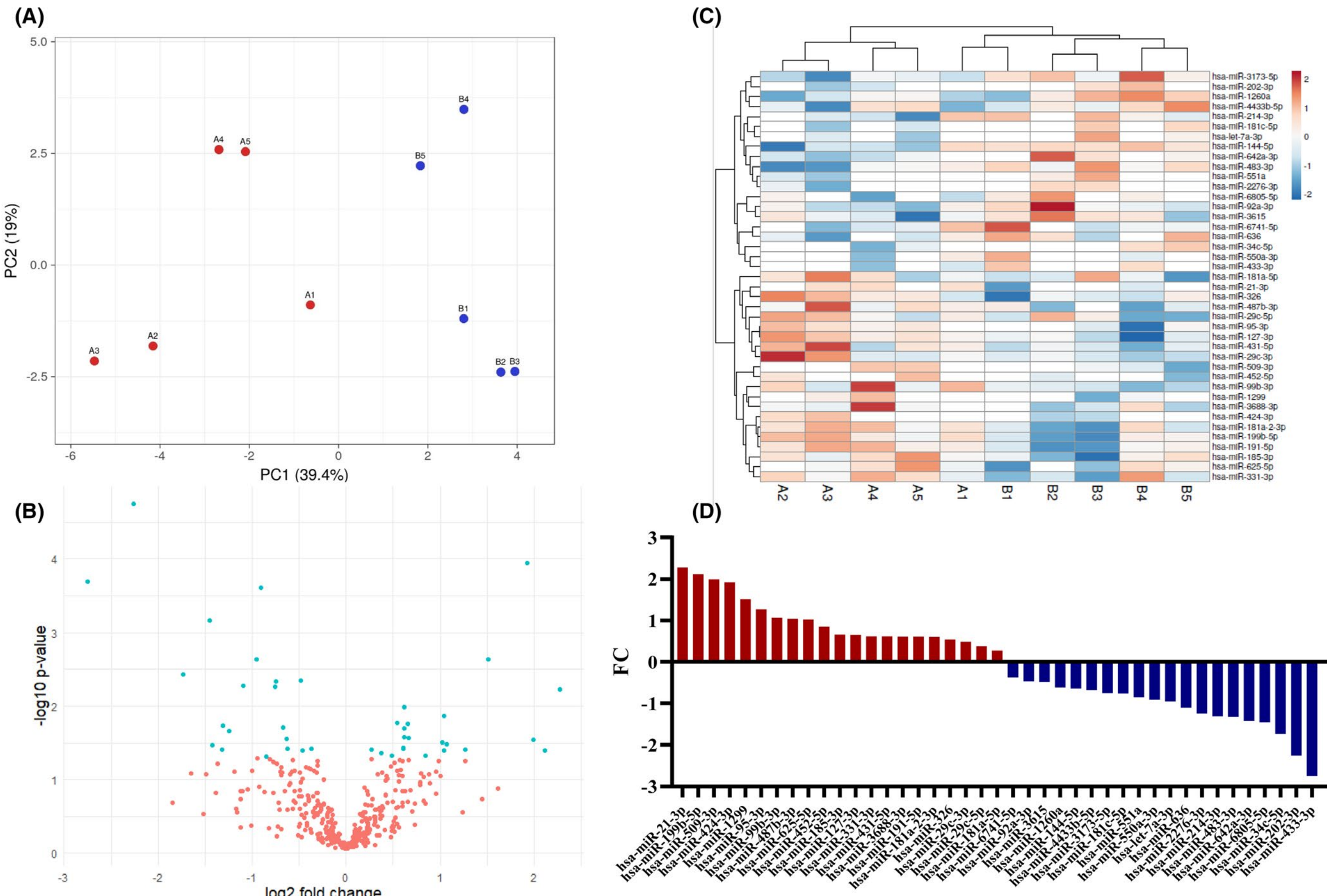


FIGURE 1 NGS-miRNAs profiling in anaphylaxis. A, The PCA exhibits similarity between samples belonging to the biologic replicas and a separation among acute (A) and basal conditions (B). B, Volcano plot shows the dispersion of total miRNAs identified by NGS with the statistically significant difference ($P < .05$) indicated in blue. C, The heatmap represents graphically the sampling and the statistically significant miRNAs identified by NGS. Units represent transformed ($\log_2$) and normalized (cyclic Loess method) abundance data obtained by NGS. D, The graphic shows the distribution by fold change (FC) of the 21 increased and the 20 decreased miRNAs. Red indicates an increase in miRNAs in the acute phase, and blue indicates a decrease in miRNAs in the acute phase

of the samples and techniques, miR-21-3p and miR-487b-3p levels revealed a significant increase in the acute phase compared with the baseline (Figure 2A,B). These data agree with the NGS results, which revealed a clear increase of their levels in acute anaphylaxis samples when compared to their baseline (Figure 2C).

3.4 | SBA of miR-21-3p and miR-487b-3p and their role in anaphylaxis

Once an increase in miR-21-3p and miR-487b-3p was confirmed in the larger cohort, we performed in silico analysis, predicting 595 and 31 different target genes, respectively. SBA was performed to further characterize their inferred role in the molecular mechanisms of the anaphylactic reaction. The miR-21-3p analysis revealed several biologic processes related to the pathophysiology of anaphylaxis specifying inflammatory response, nervous system development, and cell death and survival as the main diseases and functions. Different signaling pathways (Rac, TNFR1/2, April, and CD27) described in the canonical pathway category are part of the miR-21-3p regulatory network (Table 2).

On the other hand, the top diseases and functions standing out in the miR-487b-3p analysis were dermatologic diseases, cellular assembly, and nervous system function. These mechanisms could be related to anaphylaxis supporting a plausible role of this miRNA in the reaction. As in the previous case, the study of the canonical pathways revealed processes associated with them as histamine degradation, IL-9 signaling, and role of JAK2 in hormone-like cytokine signaling (Table 3).

3.5 | miR-21-3p increases after the incubation of ECs with anaphylaxis conditions

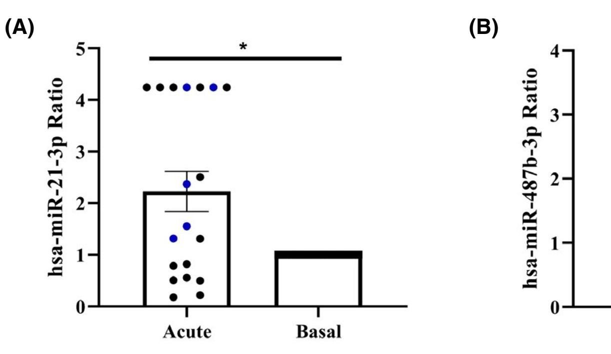
Among the variety of endotypes and phenotypes implicated in anaphylaxis, we established an in vitro serum-EC system to evaluate the functional role of miR-21-3p and miR-487b-3p in this pathologic event. Firstly, these molecules were intracellularly measured after 2 hours of incubation with anaphylactic stimuli. The determination of the UniSp6 confirmed their correct reverse transcription (Supplementary Figure 3A). Results obtained revealed that only the miR-21-3p exhibited a significant intracellular increase after the addition of a cocktail of mediators (histamine, PAF, and thrombin) or serum from the acute phase of anaphylaxis. No significant differences were found after the stimulation with serum from the basal phase (Figure 3A). Otherwise, miR-487b-3p determinations did not reveal variation in their intracellular levels after incubations (Figure 3B).

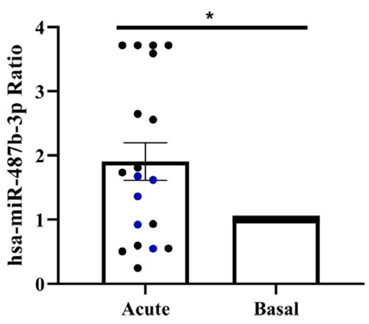
On the other hand, miR-21-3p and miR-487b-3p serum levels were measured before and after EC contact. UniSp2/4/5/6 determinations confirmed their correct extraction and reverse transcription (Supplementary Figure 3B,C). Data obtained revealed a significant increase in serum miR-21-3p serum levels from the acute phase of anaphylaxis at the end-point. Again, no significant differences were observed in basal phases, although a clear upward trend was observed (Figure 3C). As occurs in the intracellular stage, no differences were found in miR-487b-3p serum levels (Figure 3D).

4 | DISCUSSION

The aim of our study was to improve the diagnosis and enhance the knowledge about the underlying molecular mechanisms of the anaphylactic reaction. Currently, miRNAs have been investigated as biomarkers and pointed out as modulators of important altered processes in allergic diseases.[21] However, there are no data showing a clear involvement of miRNAs in human anaphylaxis. Therefore, this is the first study to report evidence about the role of serum miRNAs in this pathologic situation.

Our results show a differential miRNA profile when acute phase of anaphylaxis is compared to baseline values. In order to characterize it, a pilot-scale study was carried out with sera from 5 children, including those who revealed more severe anaphylactic features. Samples were analyzed by NGS, a transcriptomic tool to evaluate gene expression profiles and biomarker discovery.[22,23] Among the





(C)

miR	Sequence	FC	P-value
hsa-miR-21-3p	CAACACCAGUCGAUGGGCUGU	2.277	0.006
hsa-miR-487b-3p	AAUCGUACAGGGUCAUCCACUU	1.042	0.039

FIGURE 2 qPCR analysis of serum miR-21-3p and miR-487b-3p levels from 38 anaphylactic samples of 19 patients. Serum miR-21-3p (**P*: .0251) (A) and miR-487b-3p (* *P*: .0155) (B) are increased in the acute phase of anaphylaxis compared with basal. Patients included in the NGS are indicated in blue. C, miR-21-3p and miR-487-3p determination by NGS. FC: fold change

TABLE 2 miR-21-3p SBA

Top diseases and functions	Score
Cancer, inflammatory response, organismal injury and abnormalities	43
Nervous system development and function, organ morphology, organismal development	31
Cell death and survival, organismal injury and abnormalities, renal necrosis/cell death	29
Ingenuity canonical pathways	**−log(*P*-value)**
Molecular mechanisms of cancer	2.95
Rac signaling	2.82
TNFR2 signaling	2.77
April mediated signaling	2.55
B Cell–Activated factor signaling	2.5
Glutamate-dependent acid resistance	2.39
TNFR1 signaling	2.34
CD27 signaling in lymphocytes	2.29
EGF signaling	2.25
Cell cycle control of chromosomal replication	2.24
RAR activation	2.15
Pyridoxal 5'-phosphate salvage pathway	2.11
Glutamate degradation III (via 4-aminobutyrate)	2
Toll-like receptor signaling	1.98
LPS/IL-1–mediated inhibition of RXR function	1.98
BMP signaling pathway	1.89
PDGF signaling	1.88
Ceramide signaling	1.86
RANK signaling in osteoclasts	1.86
Regulation of IL-2 expression in activated and Anergic T lymphocytes	1.85

Note: Top diseases and functions and the most statistically significant canonical pathways from the Ingenuity Pathway Analysis for the miR-21-3p target genes. Activation of z-score biologic function has significantly more "increased" predictions than "decreased" predictions ($z > 0$) or vice versa ($z < 0$). *P*-value is calculated using the right-tailed Fisher exact test.

chosen panel of 41 statistically significant miRNAs detected, miR-21-3p and 487b-3p levels were validated, as increased, in the acute phase of a large population of samples from anaphylactic reactions. miR-21 has been proposed as a biomarker for children with asthma due to its increased serum levels in patients without corticosteroid therapy.[20,23] Furthermore, it targets IL-12 promoting the Th_2 polarization during the allergic airway inflammation.[24] Moreover, this miRNA is increased in human and mouse skin with allergic contact dermatitis.[25] The other miRNA of interest, miR-487b, targets IL-33, a pro-inflammatory cytokine that mediates airway inflammation mitigating the response in allergic rhinitis.[26,27] Furthermore, the analysis of fibrotic lungs revealed that miR-487b is elevated in patients compared with healthy controls.[28] In our studies, after evaluating the quality of the samples and the techniques used, the results confirmed a rise in serum levels of these miRNAs during the acute phase of anaphylaxis.

Anaphylaxis is a global health problem, and in children the main trigger is food.[29] An improved anaphylaxis diagnosis could determine its correct management and a better outcome for patients. Currently, the most widely accepted biomarker of anaphylaxis, serum tryptase, misdiagnoses more than one-third of cases, being even less accurate in those triggered by food.[8] It is the case in our cohort that none of the patients showed data above the cutoff values of tryptase reported.[7,19] This gap between tryptase values and clinical diagnosis of anaphylaxis would probably have been even larger if we had used the recent definition of anaphylaxis released by the World Allergy Organization, where bronchial symptoms alone and exposure to a known allergen are sufficient to meet the diagnosis.[14] Therefore, it is necessary to count on a more reliable laboratory molecular marker. Our results provide a promising source of candidates to investigate in future studies. However, as a limitation, the scope of our study is far from validating all of them and we postulate miR-21-3p and miR-487b-3p as possible candidates for biomarkers of anaphylaxis.

Mechanistically, very relevant questions are still not resolved in anaphylaxis. Molecular processes determining why some local allergic reactions end up becoming systemic remain unknown. Furthermore, the main molecular mechanism described in anaphylaxis points to specific IgE pathways. However, the low or null detection of specific IgE in some patients fails to explain it as the unique molecular pathway underlying reaction.[2] Indeed, other non-IgE- or

TABLE 3 miR-487b-3p SBA

Top diseases and functions	Score
Cancer, dermatological diseases and conditions, organismal injury and abnormalities	31
Cellular assembly and organization, developmental disorder, DNA replication, recombination, and repair	20
Nervous system development and function, organ morphology, tissue morphology	17
Ingenuity canonical pathways	**−log(*P*-value)**
Thyronamine and iodothyronamine metabolism	2.39
Anandamide degradation	2.39
Thyroid hormone metabolism I (via deiodination)	2.39
Acetyl-CoA biosynthesis I (Pyruvate dehydrogenase complex)	2.02
Molecular mechanisms of cancer	1.8
Dermatan sulfate degradation (Metazoa)	1.64
Histamine degradation	1.64
Oxidative ethanol degradation III	1.59
Fatty acid α-oxidation	1.57
Putrescine degradation III	1.55
Ethanol degradation IV	1.51
Tryptophan degradation X (Mammalian, via Tryptamine)	1.47
Apelin liver signaling pathway	1.46
Dopamine degradation	1.4
Ethanol degradation II	1.37
IL-9 signaling	1.36
Role of JAK2 in hormone-like cytokine signaling	1.34
Retinoate biosynthesis I	1.34
Norepinephrine and epinephrine degradation	1.33
Thyroid hormone metabolism II (via conjugation and/or degradation)	1.3

Note: Top diseases and function and the most statistically significant canonical pathways from the Ingenuity Pathway Analysis for the miR-487b-3p target genes. Activation of z-score biologic function has significantly more "increased" predictions than "decreased" predictions ($z > 0$) or vice versa ($z < 0$). *P*-value is calculated using the right-tailed Fisher exact test.

IgG-mediated mechanisms have been reported indicating that alternative molecular mechanisms (guided by miRNAs) might exist.

To determine the possible participation of miR-21-3p and miR-487b-3p in this event, an in silico analysis of their target genes was carried out. The software revealed that the main function of miR-21 was inflammation. This process is closely related to anaphylaxis because the reaction is produced by the release of inflammatory mediators.[2] Furthermore, the implication of this miRNA in inflammation and immune regulation has been already described in many other diseases.[30] The analysis of its canonical pathways showed several of them involved in these processes, among which we highlight those related to TNF receptors (CD27, April, and TNFR1/2 signaling) and Rac signaling. TNFRs participate in several processes during anaphylaxis playing a key role in enhancing endothelial permeability, thus promoting the anaphylactic shock.[31] Rac is required to stimulate the remodeling and degranulation of mast cells, the main reason why the anaphylactic reaction takes place.[2,32] On the other hand, the results obtained from miR-487b-3p revealed that the main function was dermatologic disease. The skin is the predominant organ affected during anaphylaxis, causing urticaria and/or angioedema in 85%-90% of the patients.[33] Previously, miR-487b-3p has been linked to skin lesions, although never in the allergy context.[34] Therefore, it could play a role in the inflammation and damage occurring during the reaction. Canonical pathway analysis revealed several mechanisms related to inflammation and the immune system. Different studies previously associated miR-487b-3p with these processes.[26,27] Among them, we remark the JAK2 signaling because it is required for the transduction initiated by several cytokines implicated in the pathogenesis of inflammatory skin diseases such as psoriasis and atopic dermatitis.[35] We also highlight the IL-9 signaling pathway. This molecule is a pro-inflammatory cytokine released by mast cells leading to inflammation and epithelial remodeling.[36] Our analysis shows that miR-487b-3p modulates metabolic processes and specifically histamine degradation. Histamine is one of the main allergic mediators released by mast cells and induces cutaneous flushing, airway obstruction, tachycardia, and headaches.[2] Therefore, it seems that both miR-21-3p and miR-487b-3p could participate in the molecular mechanisms of the reaction. However, future studies are necessary to confirm the specific role of these miRNAs in anaphylaxis.

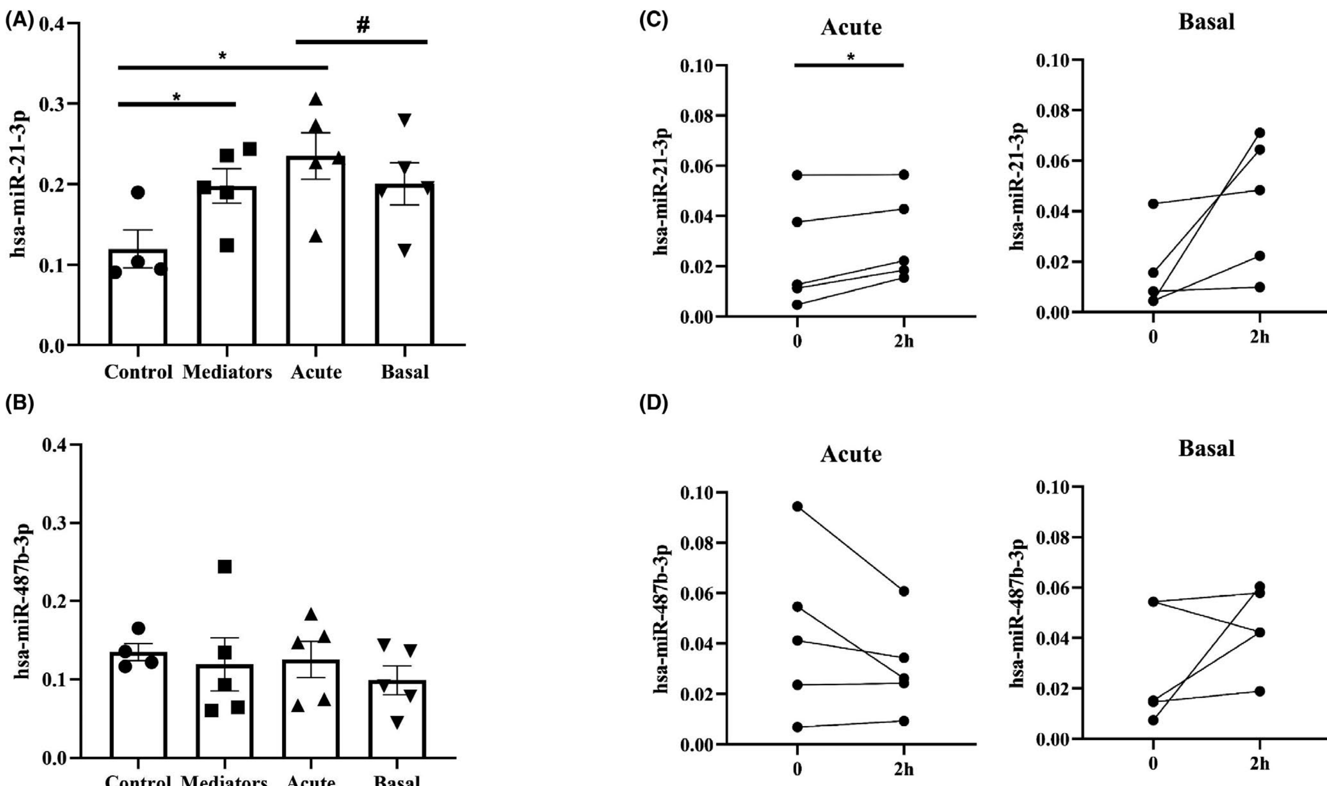


FIGURE 3 miR-21-3p and miR-487b-3p determinations in an in vitro anaphylactic serum-EC system. HMVEC-D were incubated with sera from 5 paired anaphylactic samples (acute and basal phases), a cocktail of mediators (histamine 1 µM, PAF 10 µM, and thrombin 0.5 µM), and EGM-2 medium (negative control) for 2 h. (A) miR-21-3p and (B) miR-487b-3p levels were intracellularly measured after incubation with stimuli. The cocktail of anaphylactic mediators and acute sera increased miR-21-3p values vs untreated HMVEC-D (negative control) (**P*: .0436 and **P*: .02, respectively) and between the acute phase vs baseline (#*P*: .0393). (C) miR-21-3p and (D) miR-487b-3p serum abundance was determined after EC contact. miR-21-3p serum levels increased in acute phase after a 2-h incubation with HMVEC-D vs start value (**P*: .0241). Graphics represent the mean ± SEM. Control: medium. Mediators: histamine, PAF, and thrombin. Acute: serum from acute phase of anaphylaxis. Basal: serum from basal phase of anaphylaxis. 0: start. 2 h: 2 hours (end-point)

Due to a recent study from our group that demonstrated an active participation of extracellular vesicles interacting with ECs in anaphylaxis,[16] an in vitro serum-EC system was carried out to evaluate the functional role of miR-21-3p and miR-487b-3p in this event. In order to do so, intracellular levels of both miRNAs were measured after incubation with anaphylactic conditions: cocktail of mediators and serum from patients (acute and basal phases). On the one hand, miR-487b-3p did not show any differences in its intracellular values after stimuli. On the other hand, we observed an increase in miR-21-3p levels after incubation with anaphylactic conditions. In agreement, other studies support miR-21-3p induction by pro-inflammatory agents, such as TNF-α or IL-1, in ECs.[5] Moreover, miR-21 regulates eNOS expression and it seems related to the endothelial dysfunction associated with impaired NO/cGMP molecular signaling, which is also relevant in anaphylaxis.[4,37,38] Otherwise, acute phase sera of anaphylactic patients exhibit increased abundance of miR-21-3p, but not miR-487b-3p, at the end-point of our in vitro functional system. This could be due to the release of miR-21-enriched extracellular vesicles by ECs as previously described in other cell types.[39] However, our results are far from the complete elucidation of the molecular mechanisms addressed by miR-21-3p in anaphylaxis, and therefore, further studies are still needed to clarify its relevance.

5 | CONCLUSION

In conclusion, this study has demonstrated for the first time a differential serum miRNA profile in anaphylactic children samples compared with baseline. Higher levels of miR-21-3p and miR-487b-3p during the acute phase of the reaction have been confirmed in a larger group of anaphylactic patients. In addition, in silico studies show their possible implication in inflammation and the immune regulation. Moreover, our data point to ECs as an important cellular type participating in the anaphylactic reaction increasing miR-21-3p intracellularly and contributing to its release to the extracellular environment. The data obtained intend to increase the knowledge of the molecular mechanisms of the anaphylactic reaction and to propose alternative biomarkers. However, due to the limited number of patients analyzed and sample scarcity, further research and larger study cohort are needed to determine the miRNA sensitivity and specificity. Therefore, our pioneer study facilitates a better

understanding and interpretation of the anaphylactic reaction improving the clinical approach to it and opening new directions to the participation of the miRNAs in anaphylaxis.

ACKNOWLEDGEMENTS

This research was funded by the Spanish Council Ministry of Science and Innovation (Ramón y Cajal Program RyC-12880-2013), grants from the Instituto de Salud Carlos III (PI18/00348), and FEDER Thematic Networks and Cooperative Research Centers; RETICS ARADyAL (RD16/0006/003, RD16/0006/0013, RD16/0006/0026, RD16/0006/0033). This work was also supported by the SEAIC (19_A08) and Alfonso X el Sabio University Foundations. ENB was granted by funding from the Community of Madrid included in the project FOODAL (FOODAL-CM_P2018/BAAA-4574).

CONFLICT OF INTEREST

PRR reports honoraria for consultancy and/or advisory board and/or lectures from ALK-Abello, FAES Pharma, LETI Pharma, Merck, Aimmune, Allergy Therapeutics, MEDA Pharma, and Novartis outside the submitted work. Other authors declare that they have no relevant conflicts of interest.

AUTHOR CONTRIBUTION

Emilio Nuñez-Borque: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Writing-original draft (equal). **Sergio Fernández-Bravo:** Formal analysis (equal); Investigation (equal); Writing-review & editing (equal). **Pablo Rodriguez del Rio:** Methodology (equal); Writing-review & editing (equal). **Ebrahim Mohammed Al Washali:** Methodology (equal). **David Lopez-Dominguez:** Methodology (equal); Software (equal). **Maria Dolores Gutierrez-Blazquez :** Methodology (equal); Software (equal). **Jose Julio Laguna:** Methodology (equal). **Jaime Tome-Amat:** Methodology (equal); Writing-review & editing (equal). **Julio Gallego-Delgado:** Formal analysis (equal); Validation (equal). **Alicia Gomez-Lopez:** Methodology (equal). **Diana Betancor:** Methodology (equal). **Javier Cuesta-Herranz:** Methodology (equal). **Maria Dolores Ibañez:** Methodology (equal). **Alberto Benito-Martin:** Data curation (equal); Formal analysis (equal); Methodology (equal); Writing-review & editing (equal). **Vanesa Esteban:** Conceptualization (equal); Funding acquisition (lead); Project administration (lead); Supervision (lead); Writing-original draft (equal).

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/pai.13518.

ORCID

Pablo Rodriguez Del Rio https://orcid.org/0000-0002-0783-1988
Jose Julio Laguna https://orcid.org/0000-0001-5909-8979
Jaime Tome-Amat https://orcid.org/0000-0003-4442-3649
Maria Dolores Ibañez-Sandin https://orcid.org/0000-0001-8291-5121
Alberto Benito-Martin https://orcid.org/0000-0002-0700-3891
Vanesa Esteban https://orcid.org/0000-0002-7500-6277

REFERENCES

1. Atanaskovic-Markovic M, Gomes E, Cernadas JR, et al. Diagnosis and management of drug-induced anaphylaxis in children: an EAACI position paper. *Pediatr Allergy Immunol*. 2019;30(3):269-276.
2. Reber LL, Hernandez JD, Galli SJ. The pathophysiology of anaphylaxis. *J Allergy Clin Immunol*. 2017;140(2):335-348.
3. Castells M. Diagnosis and management of anaphylaxis in precision medicine. *J Allergy Clin Immunol*. 2017;140(2):321-333.
4. Radeva MY, Waschke J. Mind the gap: mechanisms regulating the endothelial barrier. *Acta Physiol (Oxf)*. 2018;222(1):e12860.
5. Romay MC, Che N, Becker SN, et al. Regulation of NF-κB signaling by oxidized glycerophospholipid and IL-1β induced miRs-21-3p and -27a-5p in human aortic endothelial cells. *J Lipid Res*. 2015;56(1):38-50.
6. Sala-Cunill A, Cardona V, Labrador-Horrillo M, et al. Usefulness and limitations of sequential serum tryptase for the diagnosis of anaphylaxis in 102 patients. *Int Arch Allergy Immunol*. 2013;160(2):192-199.
7. Beck SC, Wilding T, Buka RJ, Baretto RL, Huissoon AP, Krishna MT. Biomarkers in human anaphylaxis: a critical appraisal of current evidence and perspectives. *Front Immunol*. 2019;10:494.
8. Lieberman P, Nicklas RA, Oppenheimer J, et al. The diagnosis and management of anaphylaxis practice parameter: 2010 update. *J Allergy Clin Immunol*. 2010;126(3):477-480.e42.
9. Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol*. 2014;15(8):509-524.
10. Jiang Q, Hao Y, Wang G, et al. Prioritization of disease microRNAs through a human phenome-microRNAome network. *BMC Syst Biol*. 2010;4(Suppl 1):S2.
11. Specjalski K, Jassem E. MicroRNAs: potential biomarkers and targets of therapy in allergic diseases? *Arch Immunol Ther Exp (Warsz)*. 2019;67(4):213-223.
12. Sampson HA, Muñoz-Furlong A, Campbell RL, et al. Second symposium on the definition and management of anaphylaxis: summary report–Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *J Allergy Clin Immunol*. 2006;117(2):391-397.
13. Brown SG. Clinical features and severity grading of anaphylaxis. *J Allergy Clin Immunol*. 2004;114(2):371-376.
14. Cardona V, Ansotegui IJ, Ebisawa M, et al. World allergy organization anaphylaxis guidance 2020. *World Allergy Organ J*. 2020;13(10):100472.
15. Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2^(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostat Bioinforma Biomath*. 2013;3(3):71-85.
16. Nuñez-Borque E, Fernandez-Bravo S, Pastor-Vargas C, et al. Proteomic profile of extracellular vesicles in anaphylaxis and their role in vascular permeability. *Allergy*. 2021. https://doi.org/10.1111/all.14792. Online ahead of print.
17. Ballesteros-Martinez C, Mendez-Barbero N, Montalvo-Yuste A, et al. Endothelial regulator of Calcineurin 1 promotes barrier integrity and modulates histamine-induced barrier dysfunction in anaphylaxis. *Front Immunol*. 2017;8:1323.
18. Metsalu T, Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. *Nucleic Acids Res*. 2015;43(W1):W566-W570.
19. Fellinger C, Hemmer W, Wöhrl S, Sesztak-Greinecker G, Jarisch R, Wantke F. Clinical characteristics and risk profile of patients with elevated baseline serum tryptase. *Allergol Immunopathol (Madr)*. 2014;42(6):544-552.

20. Anagnostou K. Anaphylaxis in children: epidemiology, risk factors and management. *Curr Pediatr Rev*. 2018;14(3):180-186.
21. Narożna B, Langwiński W, Szczepankiewicz A. Non-coding RNAs in pediatric airway diseases. *Genes (Basel)*. 2017;8(12):348.
22. Buschmann D, Haberberger A, Kirchner B, et al. Toward reliable biomarker signatures in the age of liquid biopsies - how to standardize the small RNA-Seq workflow. *Nucleic Acids Res*. 2016;44:5995-6018.
23. Elbehidy RM, Youssef DM, El-Shal AS, et al. MicroRNA-21 as a novel biomarker in diagnosis and response to therapy in asthmatic children. *Mol Immunol*. 2016;71:107-114.
24. Lu TX, Munitz A, Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *J Immunol*. 2009;182(8):4994-5002.
25. Vennegaard MT, Bonefeld CM, Hagedorn PH, et al. Allergic contact dermatitis induces upregulation of identical microRNAs in humans and mice. *Contact Dermatitis*. 2012;67(5):298-305.
26. Yamazumi Y, Sasaki O, Imamura M, et al. The RNA binding protein Mex-3B is required for IL-33 induction in the development of allergic airway inflammation. *Cell Rep*. 2016;16(9):2456-2471.
27. Liu HC, Liao Y, Liu CQ. miR-487b mitigates allergic rhinitis through inhibition of the IL-33/ST2 signaling pathway. *Eur Rev Med Pharmacol Sci*. 2018;22(23):8076-8083.
28. Milosevic J, Pandit K, Magister M, et al. Profibrotic role of miR-154 in pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 2012;47(6):879-887.
29. Grabenhenrich LB, Dölle S, Moneret-Vautrin A, et al. Anaphylaxis in children and adolescents: the European Anaphylaxis Registry. *J Allergy Clin Immunol*. 2016;137(4):1128-1137.e1.
30. Kumarswamy R, Volkmann I, Thum T. Regulation and function of miRNA-21 in health and disease. *RNA Biol*. 2011;8(5):706-713.
31. Mendez-Barbero N, Yuste-Montalvo A, Nuñez-Borque E, et al. The TNF-like weak inducer of the apoptosis/fibroblast growth factor-inducible molecule 14 axis mediates histamine and platelet-activating factor-induced subcutaneous vascular leakage and anaphylactic shock. *J Allergy Clin Immunol*. 2020;145(2):583-596.e6.
32. Sheshachalam A, Baier A, Eitzen G. The effect of Rho drugs on mast cell activation and degranulation. *J Leukoc Biol*. 2017;102(1):71-81.
33. Ring J, Behrendt H, de Weck A. History and classification of anaphylaxis. *Chem Immunol Allergy*. 2010;95:1-11.
34. Al-Eryani L, Jenkins SF, States VA, et al. miRNA expression profiles of premalignant and malignant arsenic-induced skin lesions. *PLoS One*. 2018;13(8):e0202579.
35. Fridman JS, Scherle PA, Collins R, et al. Preclinical evaluation of local JAK1 and JAK2 inhibition in cutaneous inflammation. *J Invest Dermatol*. 2011;131(9):1838-1844.
36. Kakinoki A, Kameo T, Yamashita S, Furuta K, Tanaka S. Establishment and characterization of a murine mucosal mast cell culture model. *Int J Mol Sci*. 2019;21(1):236.
37. Peñaloza E, Soto-Carrasco G, Krause BJ. MiR-21-5p directly contributes to regulating eNOS expression in human artery endothelial cells under normoxia and hypoxia. *Biochem Pharmacol*. 2020;182:114288.
38. Iannone L, Zhao L, Dubois O, et al. miR-21/DDAH1 pathway regulates pulmonary vascular responses to hypoxia. *Biochem J*. 2014;462(1):103-112.
39. An Y, Zhao J, Nie F, et al. Exosomes from adipose-derived stem cells (ADSCs) overexpressing miR-21 promote vascularization of endothelial cells. *Sci Rep*. 2019;9(1):12861.

SUPPORTING INFORMATION

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

How to cite this article: Nuñez-Borque E, Fernandez-Bravo S, Rodriguez Del Rio P, et al. Increased miR-21-3p and miR-487b-3p serum levels during anaphylactic reaction in food allergic children. *Pediatr Allergy Immunol*. 2021;32:1296–1306. https://doi.org/10.1111/pai.13518