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

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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...
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. 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 Th2 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, formed by endothelial cells (ECs), is a dynamic structure, which regulates plenty of physiologic processes. 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. 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

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

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

**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.
of pathologies. 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. 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. 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 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. 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/). 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.

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.
### Table 1: Clinical characteristics of anaphylactic patients included in this study

| Patients | Number | Age | Gender | Trigger | Symptoms | Signs | Severity | Tryptase |
|----------|--------|-----|--------|---------|----------|--------|----------|----------|
|          |        |     |        |         | Skin     | Mucous | Digestive | Respiratory | Nervous | Vascular | Hr | SatO₂ | Grade | Acute | Basal |
| 1        | 1      | 17  | F      | Milk    | E; Pr    | N; Ap   |          |          |          |          |     |       | 69  | -     | 2     | 6.93  | 5.91  |
| 2        | 2      | 11  | F      | Egg     |          | N; Ap; V; Dr | R     |          |          |          |     |       | -   | -     | 2     | 5.83  | 3.34  |
| 3        | 3      | 4   | M      | Cashew  | E        | Ag      | R        | T        |          |          |     |       | 136 | 98%   | 2     | <1    | <1    |
| 4        | 4      | 5   | F      | Roosterfish | E; Pr; U | W       |          |          |          |          |     |       | 83  | 98%   | 2     | 9.36  | 11.4  |
| 5        | 5      | 9   | F      | Milk    |          | N; Ap; V; Dr | R     |          |          |          |     |       | 83  | 99%   | 2     | 5.5   | <1    |
| 6        | 6      | 9   | M      | Egg     | E; Pr    | N; Ap; V; Dr | R        | T        |          |          |     |       | 135 | 98%   | 2     | 5.21  | 3.01  |
| 7        | 7      | 14  | F      | Egg     | E; Pr    | Ag      | N; Ap    | R        | Dz       | Hy      |     |       | 56  | 99%   | 3     | 2.6   | 2.29  |
| 8        | 8      | 11  | F      | Milk    | U        | Ag      | R; W     |          |          |          |     |       | 89  | 97%   | 2     | 3.81  | 3.31  |
| 9        | 9      | 15  | M      | Cheese  | E        |          | R; W     |          |          |          |     |       | 57  | 96%   | 2     | 8.03  | 5.69  |
| 10       | 10     | 13  | F      | Milk    |          | Ap      | R; W     |          |          |          |     |       | 77  | 98%   | 2     | 5.04  | 2.98  |
| 11       | 11     | 5   | M      | Milk    | E        | Ap; V   | Dy       | Hy       |          |          |     |       | 85  | 94%   | 3     | 7.83  | 4.97  |
| 12       | 12     | 14  | M      | Egg     | E; Pr    | Ag      | N; Ap; V; Dr | R     |          |          |     |       | 81  | 98%   | 2     | 2.88  | 2.44  |
| 13       | 13     | 10  | F      | Egg     | E; Pr    | Ag      | W       |          |          |          |     |       | 117 | -     | 2     | 3.89  | 3.78  |
| 14       | 14     | 14  | F      | Nut     | U        | Ag      | Cg       | Dz       |          |          |     |       | 96  | 99%   | 2     | 6.63  | 5.65  |
| 15       | 15     | 7   | M      | Roosterfish |        | Ag      |          |          |          |          |     |       | 85  | 98%   | 2     | 3.84  | 2.53  |
| 16       | 16     | 14  | F      | Milk    | U        | N; Ap   | R; W     |          |          |          |     |       | 107 | 97%   | 2     | 8.74  | 1.72  |
| 17       | 17     | 11  | F      | Milk    | E        | Ag      | N; Ap    | R; Cg    |          |          |     |       | 100 | 99%   | 2     | 8.57  | 7.05  |
| 18       | 18     | 15  | M      | Peanut  |          | Ap; V   | Dy       |          |          |          |     |       | 93  | 99%   | 2     | 3.03  | 1.73  |
| 19       | 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 SatO₂ (oxygen saturation). Tryptase values correspond to ng/mL.

*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
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. 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. Among the

| 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.
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 Th2 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.3 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 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.
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. Furthermore, the implication of this miRNA in inflammation and immune regulation has been already described in many other diseases. 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. Rac is required to stimulate the remodeling and degranulation of mast cells, the main reason why the anaphylactic reaction takes place. 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. Previously, miR-487b-3p has been linked to skin lesions, although never in the allergy context. 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. 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. 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. 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. 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.

| TABLE 3 miR-487b-3p SBA | 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.
Due to a recent study from our group that demonstrated an active participation of extracellular vesicles interacting with ECs in anaphylaxis, 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 acute sera. 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, in an 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. 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. 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. 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 of anaphylaxis and its potential as a biomarker.
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.

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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 Rodríguez del Rio: Methodology (equal); Writing-review & editing (equal). Emmanuel Mohammed Al Washali: Methodology (equal). David Lopez-Domínguez: Methodology (equal); Software (equal). María 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-Martín: 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).

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SUPPORTING INFORMATION

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

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