

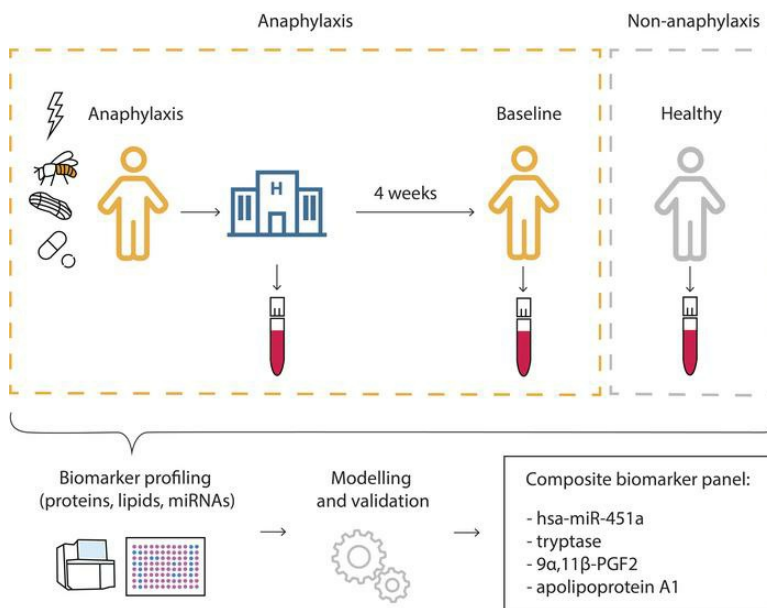
Serological profiling reveals hsa-miR-451a as a possible biomarker of anaphylaxis

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Serological profiling reveals hsa-miR-451a as a possible biomarker of anaphylaxis

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19 Abstract

20 **Background:** There is a need to support the diagnosis of anaphylaxis by objective markers.
21 miRNAs are promising non-coding RNA species that may serve as serological biomarkers,
22 but their use in diagnosing anaphylaxis has not been systematically studied. We aimed to
23 comprehensively investigate serum biomarker profiles (proteins, lipids, and miRNAs) to
24 support the diagnosis of anaphylaxis.

25 **Methods:** Adult patients admitted to the emergency room with a diagnosis of anaphylaxis
26 (< 3 hours) were included. Blood samples were taken upon emergency room arrival and
27 one month later.

28 **Results:** Next-generation sequencing of 18 samples (6 anaphylaxis patients in acute and
29 non-acute condition, respectively, and 6 healthy controls) identified hsa-miR-451a to be
30 elevated during anaphylaxis and verified by RT-qPCR in the remaining cohort. The random
31 forest classifier enabled us to classify anaphylaxis with high accuracy using a composite
32 model. We identified tryptase, 9 α ,11 β -PGF₂, apolipoprotein A1, and hsa-miR-451a as
33 serological biomarkers of anaphylaxis. These predictors qualified as serological biomarkers
34 individually but performed better in combination.

35 **Conclusion:** Unexpectedly, hsa-miR-451a was identified as the most relevant biomarker in
36 our data set. We were also able to distinguish between patients with a history of
37 anaphylaxis and healthy individuals with higher accuracy than any other available model.
38 Future studies will need to verify miRNA biomarker utility in real-life clinical settings.

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43

44 Introduction

45 Anaphylaxis is a type I hypersensitivity reaction that is potentially life-threatening if not
46 adequately treated. Although the simultaneous onset of skin symptoms and hypotension
47 (in patients with a history of allergic reactions) suggests an anaphylactic episode –
48 reactions may present atypically as skin symptoms may not be present in up to 20% of
49 patients (1, 2). To more confidently administer adrenaline as the first-line treatment in
50 cases where the elicitor of anaphylaxis is unknown and the diagnosis uncertain - there is a
51 high medical need for objective laboratory parameters supporting the diagnosis of
52 anaphylaxis (preferably using easy to acquire biological samples i.e., blood).

53 Currently, total mast cell tryptase is the only laboratory parameter routinely used for the
54 diagnosis of anaphylaxis, but its moderate sensitivity and specificity require improvement
55 (3). Moreover, the values of tryptase during acute reaction need to be related to their
56 baseline levels in the same individual due to substantial inter-individual variability even on
57 baseline (4). In addition, the relatively late diagnostic window for serum tryptase between
58 30 – 120 minutes after the onset of first anaphylaxis symptoms is too late concerning the
59 necessity of rapid treatment initiation. Therefore multiple efforts have been undertaken in
60 identifying other potential biomarkers of anaphylaxis (5–8)

61 miRNAs are small non-coding RNAs that regulate gene expression usually by silencing
62 transcription (9). Their use as serological biomarkers has been proposed in various
63 diseases (10). Data on the role of miRNA in human anaphylaxis is largely unknown. A
64 recent study in pediatric oral food provocations of 4 children reported differential

expression of several miRNAs during the reaction, indicating their potential to assist in the diagnosis of anaphylaxis (11). Since several miRNAs were shown to play a role in acute asthma via airway inflammation and hyperresponsiveness (12), we hypothesized that miRNA secretion may also occur in hypersensitivity reactions.

The aim of this study was therefore to identify biomarkers predictive of anaphylaxis with a special emphasis on miRNAs.

Results

Study cohort characteristics

We prospectively recruited 81 patients from the emergency room (ER) with a suspected diagnosis of anaphylaxis. Out of this cohort 31 patients had to be excluded from the analysis due to low severity, or prolonged time (>3 hours) from the onset of the first symptoms till blood draw in the ER or due to not fulfilling the diagnostic criteria for anaphylaxis. Second sampling during a diagnostic visit was performed in 24 out of 47 anaphylactic patients at the Allergy and Immunology Division (Figure 1A). 24 healthy individuals without a history of hypersensitivity reactions were recruited as controls. The mean age of patients and healthy controls was 48.77 (median = 52) and 46.26 (median = 40) years respectively and the sex distribution was comparable (Figure 1B-C). The majority of patients were White (European 85.11%, five, 10.64% were of Middle East descent), and two (4.26%) were Asian (Far East). The classification was made by the investigators. Table S1 summarizes the studied individuals.

The majority of cases in our cohort were venom-induced anaphylaxis (VIA, n = 22) and food-induced anaphylaxis (FIA, n = 19), followed by drugs (DIA, n = 3), and three cases of idiopathic anaphylaxis. (Figure 1D). VIA did not show significantly more severe reactions, classified as Ring and Messmer (13) grade III, than FIA ($p = .089$). There were no significant differences in severity between sexes (data not shown).

Skin symptoms were most prevalent regardless of elicitor. Symptoms of VIA were more often associated with the cardiovascular system ($p = .036$) whereas there was a tendency of greater gastrointestinal and respiratory involvement in FIA than in VIA without reaching significance ($p = .386$ and $p = .204$ respectively). There were no cases of respiratory or cardiac arrest (Figure 1E).

Identification of 19 miRNA candidates via next-generation sequencing

The initial screening of 6 anaphylactic patients (3 VIA and 3 FIA) resulted in successful labeling and counting of 2656 miRNAs after removing entities with less than 100 counts.

We observed differential expression of 19 miRNAs, with p values $< .05$ after FDR adjustment using Benjamini-Hochberg correction, between samples taken from the patients in anaphylaxis and their respective baselines. (Figure 2A). The samples from an acute reaction clustered together when unsupervised clustering was performed, separating them from healthy and baseline as indicated in the dendrogram (Figure 2A).

Principal component analysis showed some but no precise differentiation across the groups (regardless of the number of differentially expressed miRNAs, Figure 2B). Based on

105 the differential expression analysis (with the DESeq2 package for R (14)) we selected 5
106 miRNAs as potential candidate biomarkers for further study: hsa-miR-451a, hsa-miR-143-
107 3p, hsa-miR-486-5p, hsa-miR-25-3p, and hsa-miR-484, performing RT-qPCR validation on
108 the sera of patients who provided baseline samples.

109 hsa-miR-451a and hsa-miR-486-5p showed a significant difference in Δ_{Ct} values between
110 groups and it was highly enhanced in anaphylaxis samples (Figure 2D). Notably, a control
111 group of patients with atopic dermatitis, and no medical history of anaphylaxis, showed
112 similar levels of hsa-miR-451a to healthy controls (Figure S6). Conversely, hsa-miR-25-3p
113 and hsa-miR-484 showed significantly higher Δ_{Ct} in both anaphylaxis and baseline samples
114 when compared to healthy controls, but they did not differentiate between anaphylaxis and
115 baseline samples of the same patients. hsa-miR-143-3p did not show significant differences
116 across the groups (Figure S1).

117 **Patterns of serological biomarkers in anaphylaxis - a collective view**

118 We observed elevated tryptase in anaphylaxis compared to baseline and healthy control
119 samples (Figure 3). Moreover, we were able to confirm our recently identified markers 11 β -
120 prostaglandin F₂ α (PGF₂, increase) and apolipoprotein A1 (ApoA1, decrease), in this
121 independent cohort (6, 8). In addition, we identified arachidonic acid (AA) to be
122 significantly decreased in our anaphylaxis samples when compared to healthy controls
123 (Figure 3D). No significant differences were observed for other potentially relevant
124 proteins including cysteinyl leukotriene (cys-LT; Figure 3E), CCL13, CCL27, Apolipoprotein

125 E (ApoE), CCL17, eosinophil cationic protein (ECP), or chitinase-3-like protein 1 (YKL-40),
126 as shown in Figure S1A – F.

127 To better understand the biomarker profiles during anaphylaxis we stratified the patients
128 according to the elicitor and grade of reaction. Ring and Messmer grade III reactions mostly
129 showed greater differences in the measured biomarkers when compared to healthy
130 controls and baseline (Figure S2A). Biomarker levels were not altered by adrenaline
131 therapy, except for AA, which was lower in patients who did not receive adrenaline (Figure
132 S2B).

133 hsa-miR-451a was higher in patients undergoing anaphylaxis due to insect venom when
134 compared to food-induced anaphylaxis (Figure S2C). Baseline levels of hsa-miR-25-3p were
135 elevated in VIA both on baseline and during anaphylaxis when compared to FIA. YKL-40
136 and AA showed the expected trends in VIA but not in FIA, despite comparable levels in the
137 baseline of both groups (Figure S2C). Levels of hsa-miR-25-3p were elevated on baseline
138 and anaphylaxis in VIA when compared to FIA.

139 Cross-sectional time analysis of the biomarker levels in various time intervals from the
140 studied cohort indicated that tryptase and PGF2 levels peaked (and ApoA1 bottomed)
141 between 60 – 100 minutes after anaphylaxis onset. The hsa-miR451a showed a sideways
142 trend in time, with the highest value observed at around 60 minutes. AA levels post
143 anaphylaxis were lower in cases when the time interval from anaphylaxis onset to blood
144 draw was extended. The highest levels of hsa-miR-25-3p were observed 120 minutes after
145 the onset of anaphylaxis symptoms (Figure S3A – F).

Random forest accurately classified samples using a set of biomarkers

To assess the diagnostic value of the distinct biomarkers, we performed a classification of samples using the random forest algorithm. In the initial model, all candidates were supplied as predictors and the importance of each predictor was calculated, indicating hsa-miR-451a as the most important predictor (Figure 4A). To minimize the set of predictors, we performed recursive feature elimination and arrived at a model with 4 predictors having the highest accuracy (Figure 4C). Hyperparameter tweaking optimally indicated 2 randomly selected variables for each split (Figure S4B). We performed 10 runs of 10-fold repeated cross-validation using the caret package for R (15) and arrived at 0.93 area under the receiver operating characteristic curve for the top 4 predictors (out of bag error (OOB) = 12%, Figure S4A). This was 0.15 higher when compared to a model where random forest used 3 predictors for classification: tryptase, PGF2, and ApoA1. Thus, the addition of hsa-miR-451a increased the precision to detect an acute reaction.

To validate the model, we used the remaining 30% of randomly pre-selected observations (n = 9 during anaphylaxis; n = 8 on baseline). The final model correctly classified 16 cases (94.12%). Only one sample originating from the baseline group was incorrectly assigned as anaphylaxis (Figure 4B).

Enrichment analysis of the differentially expressed miRNAs

We used two computational tools (miEAA2.0 (16), MIENTURNET (17)) and FANTOM5 mammalian expression miRNA atlas (18) to predict the pathomechanistic relevance of

166 selected miRNAs in anaphylaxis. The miEAA2.0 analysis yielded 163 enriched
167 subcategories (out of over 13,000) in 7 categories out of 19 used. Figure 5A summarizes
168 the first 25 most significantly enriched subcategories.

169 Most of the enriched subcategories came from Gene Ontology (miRWalk) category,
170 indicating enrichment in protein transport, carbohydrate metabolism, oxidative stress,
171 inflammation through the TNF- α signaling pathway, and cell activation with the PI3 kinase
172 pathway. Regarding the localization of miRNAs based on the RNALocate2.0 database (19)
173 we saw enrichment in the exosome subcategory, as expected.

174 Concurrent analysis was performed using the MIENTURNET online tool (17) with 19
175 differentially expressed miRNAs (2 minimum miRNA per gene target and FDR < .05) using
176 the miRTarBase database (20). Target prediction indicated highly significant involvement
177 of trans-Golgi network proteins (Golgin A8 A and B, vacuolar protein sorting-associated
178 protein) as well as signaling (Protein Phosphatase 1 Regulatory Subunit 37), carbohydrate
179 metabolic processes (Hexokinase 2), and transcription regulatory processes (Zinc Finger
180 Protein 264, RNA Binding Motif Protein 27) (Figure S5). Target genes identified in
181 miRTarBase overlapped with the pathways identified by miEAA2.0 analysis.

182 Reactome (21) pathway analysis with the predicted miRNA target genes identified
183 enrichment in apoptosis, IL-4 and IL-13 signaling, activation of metalloproteases, and
184 multiple tyrosine kinases signaling (Figure 5B).

The cellular origin of the 12 most differentially expressed miRNAs was determined based on the data from the FANTOM5 Project (18) providing expression levels of known miRNAs in 118 different cell types. No expression of hsa-miR-3178 and hsa-miR-10400-5p was detected. Neutrophils, monocytes, and B-cells were the primary source of the most differentially expressed miRNAs and hsa-miR-451a was primarily expressed in neutrophils.

miRNAs identified patients prone to anaphylaxis

Parameters that distinguish patients at the risk of developing anaphylaxis remain unknown but are highly desirable for risk assessment and the implementation of prophylactic measures or even for retrospective diagnosis.

In the preceding data (Figure 6), we fortuitously observed that in addition to the steep increase in hsa-miR-451a during anaphylaxis, other entities were enhanced in baseline measurements vis-à-vis healthy controls. Since this provided a strong hint that miRNAs may serve in the identification of patients at risk, we employed the same procedure as above to examine this possibility. This was performed, by designing a random forest model. Indeed, the combination of 5 predictors (hsa-miR-484, hsa-miR-25-3p, hsa-miR-451a, ApoE, YKL-40) resulted in a random forest model showing good accuracy to differentiate between patients prone to anaphylaxis (i.e. baseline samples, from patients that had anaphylaxis ~4 weeks prior to sampling - B) and healthy controls (HC). The model was greatly improved against the reference model (tryptase, PGF2, ApoA1) with an area under ROC curve of 0.82 versus 0.46 respectively (0.36 difference, Figure 6D).

205 Though the predictive capacity of the model was somewhat limited due to the lower
206 number of observations used for training (n =22, OOB = 18.18%), the model still correctly
207 assigned 66.67% of cases (Figure 6B) on the 30% of previously unseen data. While more
208 data is needed, this is arguably the first successful step towards the detection of a
209 biomarker panel to assist in the identification of patients prone to anaphylaxis.

210 Discussion

211 This study finds hsa-miR-451a as a novel and robust biomarker of anaphylaxis which may
212 improve the diagnostic accuracy, especially when combined with other available
213 biomarkers. In our prospective clinical observational study, we determined a higher
214 expression of hsa-miR-451a in serum of patients with anaphylaxis when compared to
215 healthy controls and non-acute sera and were able to model its diagnostic capacity using a
216 random forest classifier. Importantly, we achieved a high classification accuracy when the
217 model was tested on previously unseen real-life data.

218 The studied cohort adequately represented anaphylaxis cases in Europe. Insect venom was
219 the most prevalent elicitor of anaphylaxis in our cohort, corresponding to the previously
220 published data for Europe (22). Although US epidemiological data indicate medication
221 followed by food as the primary triggers of anaphylaxis (23), food anaphylaxis is most
222 prevalent in children (24) who were not included in this study. In accordance with our
223 data, the clinical presentation of anaphylaxis with cardiovascular symptoms is known to be
224 more prevalent in VIA than in FIA (1, 25) and the frequency of grade IV cases (Ring &

225 Messmer scale) is known to be low (26). We did not include cases with Ring & Messmer
226 grade I, as they did not meet the definition of anaphylaxis by FAAN/NIAID (27). In line with
227 previous reports, we observed more severe cases in VIA (1). One of the limitations of this
228 study was the lack of anaphylaxis due to drugs (we had only one paired case). Therefore,
229 this may be of interest for future studies.

230 Nevertheless, acquiring samples from patients in anaphylaxis is difficult as the main
231 concern is to provide immediate therapy to a patient with a potentially lethal allergic
232 reaction. Therefore, in some cases, a low amount or incorrect biomaterial was sampled
233 upon the ER visit. Fortunately, a substantial number of patients complied with a follow-up
234 visit to our Allergy and Immunology Division ~4 weeks post-reaction, so that patient-
235 matched serum samples during anaphylaxis and baseline were available for the study. This
236 was not possible with 48% of patients, yet we were able to use unpaired samples for the
237 random forest modeling, which allowed us to achieve good model accuracy. It needs to be
238 pointed out that in real-life scenarios, patient-matched baseline sera are unavailable in
239 most situations, so that biomarker models, that do not require the matched non-acute
240 serum from patients will best serve in the clinic.

241 This study did not only explore a new molecular category, rarely investigated in the context
242 of anaphylaxis, i.e., miRNAs, but it also comprehensively assessed them in regard to other
243 high confidence biomarkers to grade their utility. The selection of protein and lipid
244 biomarkers was made based on literature research and our findings.

245 Tryptase has been extensively studied as a biomarker of anaphylaxis. The gold standard
246 involves the measurement of serum total tryptase during an acute phase followed by a
247 baseline measurement (≥ 24 h) (28). Although tryptase levels correlate with the severity of
248 anaphylaxis (29) and this is also visible in our data, two similarly designed studies
249 conducted in emergency departments failed to confirm a predictive role of tryptase or
250 histamine in the diagnosis of anaphylaxis (30, 31). Nonetheless, tryptase is the best-studied
251 entity already in clinical use despite its limitations. However, we previously identified new
252 candidates, demonstrating the predictive power of PGF₂, cys-LTs, ApoA1, and ApoE in the
253 diagnosis of anaphylaxis (6, 8). Notably, PGF₂ and ApoA1 were not only reproduced in our
254 current, broader study using an independent cohort, but they turned out to be in the top 4
255 classifiers of the random forest, and thereby, part of the best biomarker composite model
256 to predict anaphylaxis. AA was selected because it is a precursor for PGF₂ synthesis (32).
257 Upon anaphylaxis, levels of AA were expected to decrease as it is metabolized to
258 prostaglandins, leukotrienes, and thromboxanes, and this was indeed observed. The other
259 candidates included were mainly based on their utility in other atopic or mast cell driven
260 diseases. For example, CCL27 (CTAK) is secreted in the skin and seemed to be associated
261 with urticaria (33). Although skin symptoms were highly prevalent during anaphylaxis in
262 our cohort, there were only a few samples with an increase in CCL27 in serum. CCL13
263 (MCP-4) has been implicated in inflammatory processes in asthma (34, 35), CCL17 (TARC)
264 is a biomarker of atopic dermatitis severity with a reported case of transient increase after
265 food-induced anaphylaxis (36). YKL-40 has been implicated in atopic diseases (allergic
266 rhinitis (37), asthma (38, 39), and food allergy (40)). Raised ECP was described in food

267 anaphylaxis (41) and food provocation (42). However, none of them qualified as a potential
268 biomarker of anaphylaxis in our study.

269 ApoA1 was discovered through a proteomics screen in our previous study (8). Since the
270 strategy proved successful in biomarker exploration, we reapplied an analogous unbiased
271 approach to exploit the untapped potential of miRNAs. The use of paired biosamples
272 (during anaphylaxis and baseline) allowed us to achieve decent group discrimination in
273 unsupervised hierarchical clustering, despite the relatively low number of sequenced
274 samples. By using stringent differential expression analysis and state-of-the-art tools (14,
275 43), we were able to identify 19 significantly expressed miRNAs and tested extensively 5
276 promising candidates by RT-qPCR in the whole cohort. The NGS data could be reproduced
277 on a larger cohort indicating the usefulness of this approach for the identification of
278 serological biomarkers.

279 During modeling, we used a reference random forest model with 3 predictors that have
280 previously shown predictive potential in diagnosing anaphylaxis. In our previous
281 publication, a composite linear model using ApoA1 and PGF2 (8) was able to discriminate
282 between anaphylaxis cases and healthy controls (but the results were not cross-validated).
283 Therefore, we benchmarked our modeling to a presumably best performing composite
284 model using ApoA1, tryptase and PGF2. The addition of hsa-miR-451a significantly
285 improved the model accuracy and proved valid upon testing with previously unseen data.
286 Notably, hsa-miR-451a was more important as a predictor for the random forest classifier
287 than tryptase.

288 Regarding cellular origin, hsa-miR-451a was identified to be expressed by neutrophils and
289 monocytes, based on the differentially expressed miRNAs in FANTOM5 (18). In addition,
290 hsa-miR-451a is known to be expressed by erythrocytes (44–47), which were not included
291 in the FANTOM5 cell atlas. Based on that, the increase in serum levels of hsa-miR-451a
292 might be: 1) co-released into serum along with cellular activation and degranulation
293 processes; 2) linked to the degradation or release from erythrocytes upon anaphylaxis.
294 Future studies will have to address the major cellular sources operative in anaphylaxis and
295 mechanisms of release (direct versus effector cell driven, e.g., via mast cells or basophil
296 activation). Although, we demonstrated that hsa-miR-451a was not increased in other
297 atopic disease (atopic dermatitis) it would be crucial to verify its specificity when
298 compared to other shock syndromes (i.e., myocardial infarction, sepsis and hypotensive
299 shock).

300 An intriguing and unexpected outcome of this study was the differential miRNA profile in
301 anaphylaxis prone patients (anaphylaxis patients sampled during baseline) versus healthy
302 individuals. Not all individuals with an increased sIgE to a particular allergen respond to
303 the allergen in question and the severity of the reaction and organ involvement are
304 likewise unpredictable by sIgE levels or other diagnostic tests such as skin prick test or
305 basophil activation test (48). Most importantly, the use of skin prick tests or sIgE failed to
306 be predictive of reaction severity in food allergic children (49). Currently, the only usable
307 predictors of future anaphylaxis severity are clinical symptoms of the previous reaction
308 and the presence of the cofactors of anaphylaxis (50). The European Guidelines for the

309 Management of Anaphylaxis (PRACTALL) underline the need for an objective, predictive
310 biomarker of future reaction severity (51). At the moment there are no point of care tests
311 available for anaphylaxis and the first step towards achieving this goal is to confirm
312 miRNAs value as a diagnostic tool in clinical setting. With the rapid development in nucleic
313 acid tests seen during the COVID-19 pandemic (52) we can expect increasing use of similar
314 technology for the diagnosis of other diseases.

315 It is therefore a major unmet clinical need to identify those patients who are at risk of
316 severe hypersensitivity reactions based on serum biomarkers alone. This knowledge can be
317 used to increase patients' awareness, provide additional prophylactic measures, and even
318 to help in the differential diagnosis of anaphylaxis retrospectively using serum. So far, no
319 single or composite biomarkers are available to meet this aim. Here we find that our
320 proposed composite biomarker (hsa-miR484, hsa-miR-25-3p, hsa-miR-451a, ApoE, and
321 YKL-40) could distinguish between anaphylaxis prone and healthy with a higher accuracy
322 than any other available model. Interestingly, the miRNAs that distinguish anaphylaxis
323 prone from healthy individuals were different than the aforementioned hsa-miR-451a, the
324 latter best suited to diagnose acute symptom precipitation. This was further emphasized by
325 random forest analysis whereby the most significant predictors of the two independent
326 questions (i.e. to diagnose acute anaphylaxis and to identify patients at risk) did barely
327 overlap (compare Figure 4 with Figure 6). In fact, of all biomarkers measured, only miRNAs
328 turned out to be reasonable predictors of anaphylaxis risk, highlighting the major potential
329 of this class of biomolecules. Thus, the results can serve as a basis to implement miRNAs in

330 the analytic make-up to diagnose an acute anaphylaxis attack. Yet more, they also provide a
331 rationale for future research to prospectively identify patients at risk based on serological
332 miRNA profiles alone.

333 **Methods**

334 **Inclusion criteria, sample collection and storage**

335 We obtained serum samples from adult patients undergoing anaphylaxis who were
336 admitted to the ER and provided written informed consent, as well as control (baseline)
337 serum samples at least 1 month after anaphylaxis. The diagnosis of anaphylaxis was made
338 according to the National Institute of Allergy and Infectious Disease/Food Allergy and
339 Anaphylaxis Network criteria (27). We also acquired serum samples from healthy
340 individuals who did not report a history of allergic diseases as well as a second control
341 group consisting of atopic dermatitis patients without previous medical history of
342 anaphylaxis. After collecting the whole blood, it was left undisturbed at room temperature
343 for 20 to 30 minutes to allow blood to clot. Samples were then centrifuged in a pre-chilled
344 centrifuge with a horizontal rotor (swing-out head) for 5 minutes at 1500 g at 4°C,
345 aliquoted, and stored at -80°C.

346 **Protein and lipid biomarker measurement**

347 Human serum samples were analyzed using ELISA kits provided by R&D Systems,
348 Minneapolis, USA (human CCL17, DY364; human CCL13, DY327; human CCL27, DY376;

human YKL-40, DC3L10), Cusabio Technology, Houston, Texas (Human Arachidonic Acid, CSB-E09040h; Human Eosinophilic Cationic Protein, CSB-E11729h), Cayman Chemical Company, Ann Arbor, USA (11β -Prostaglandin F 2α , 516521; Cysteinyl Leukotriene, 500390-96), Abcam, Cambridge, UK (Human Apolipoprotein A1, ab108804), Raybiotech Life, Norcross, USA (Human ApoE, ELH-ApoE) following the manufacturer's protocol. The total concentration of tryptase in serum was measured by ImmunoCAP (Thermo Fisher Scientific, Waltham, USA).

Serum miRNA extraction and profiling by NGS sequencing

Circulating miRNAs were isolated from 200 μ l serum by the miRNeasy Serum/Plasma kit (Qiagen, 217184) and quantified with Agilent 2100 Bioanalyzer system. Library prep was performed using the QIASeq miRNA library kit (Qiagen, 331502) according to the manufacturer's instructions. Briefly, a total of 5 μ l RNA was used for miRNA library preparation. After ligation of the RNA 3'- and the RNA 5' adapter, universal cDNA synthesis with UMI assignment, cDNA cleanup, library amplification, and library cleanup were performed. Library preparation quality control was performed using TapeStation (Agilent Technologies).

Sequencing was performed on Illumina miSeq Platform. Samples from 6 patients undergoing anaphylaxis were compared to their corresponding sera taken outside of an allergic reaction (i.e., baseline). The obtained reads were initially trimmed with cutadapt version 2.4 (53) using the first 10 bases of the Qiagen 3' adapter sequence. Reads shorter than 10 bases after trimming were removed. Over 96% of reads in each sample contained

370 an adapter. Reads were aligned to the mirBase (release 22.1) human micro-RNA sequences
371 (54) and counted using the Mirdeep2 package (55) (v. 2.0.1.2). To this end, reads were
372 trimmed as described above, converted to the FASTA format and collapsed using the
373 collapse_reads_md.pl program. Quantification was performed with the quantifier.pl
374 program. Quality control was performed using fastQC v. 0.11.8 (56), RNA-Seqc, and
375 dupradar (57).

376 **Data availability**

377 The sequencing data presented in this study have been deposited in the European
378 Nucleotide Archive (ENA) under accession number PRJEB50710.

379 **Differential expression analysis**

380 Differential expression of miRNA sequencing data from 6 paired samples was analyzed
381 using the DESeq2 package (14) for R (58), with log fold change shrinkage using the Ashr
382 algorithm (59). Stringent false discovery rate adjustment was applied for the p values (60).
383 A heatmap of differentially expressed miRNA was produced using the heatmaply
384 package(61), with row wise-scaling and automatic clustering using euclidean distances.

385 **Validation of miRNA using quantitative RT-PCR**

386 The differentially expressed miRNAs were further validated using reverse transcription
387 quantitative real-time PCR (RT-qPCR). Briefly, total RNA was isolated from 200 µl serum by
388 the miRNeasy Serum/Plasma kit (Qiagen, 217184) according to the manufacturer's
389 instructions. In addition, 3.5 µl miRNeasy serum/plasma spike-in control (Qiagen, 219610)

390 at 1.6×10^8 copies/ μ l was added to each sample. The total RNA was reverse transcribed
391 using miRCURY LNA RNA kit (Qiagen, 339340) that generates universal cDNA templates
392 for all miRNAs present in the sample. The synthetic spike-in (UniSp6, Qiagen, 339340) was
393 added to each sample, and the reaction was performed in the GeneTouch thermal cycle
394 (Bioer). Then, miRNA-specific quantification was performed using miRCURY LNA SYBR
395 Green kit (Qiagen, 339347) according the manufacturer's instructions. The expression of
396 target miRNAs was normalized to the cel-miR-39-3p synthetic spike-in added during total
397 RNA extraction.

398 **Classification model**

399 The supervised machine learning classification model was done using a random forest
400 algorithm with the help of the caret package (15). All samples with complete observations
401 (without missing values) were included in the training set and divided into anaphylaxis and
402 baseline groups (the latter contained healthy controls and patients' baseline samples).
403 Features for the final random forest model were selected by a recursive feature elimination
404 algorithm on the set of initially available biomarkers. Variable importance was derived
405 from the initial random forest model, which included all predictors. Hypeparameter
406 optimization was internally performed by the caret package to identify the optimal number
407 of randomly drawn candidate variables at each decision tree split, based on the Out-of-Bag
408 error estimate (62). The model fitness was calculated using 10 sets of 10-fold repeated
409 cross-validation.

410 Target genes prediction and functional enrichment

411 The functional miRNA set enrichment analysis was performed using the miEAA2.0 online
412 tool (16). Briefly: 1) an ordered set of 500 most differentially expressed miRNAs based on
413 the output from DESeq2 (adjusted p-values of the 6 screened anaphylaxis patients and
414 their corresponding baseline samples) was provided for the miEAA2.0. 2) Subsequently the
415 algorithm cross-referenced known and predicted miRNA-gene interaction targets
416 restricting the significance level to .005 and the minimum required miRNA hits per sub-
417 category of 5. We used FDR (Benjamini-Hochberg) p value adjustment for the whole set of
418 analyses in all categories. 3) The resulting miRNA list for miRNA Set Enrichment Analysis
419 (miRSEA) in all available 19 default gene-set categories including miRTarBase and
420 miRWalk - for gene ontology, Cell type specific atlas, Immune Cells gene sets, Localization -
421 RNALocate and pathways based on miRWalk database). 25 most significantly enriched sets
422 were subsequently plotted to indicate the presence (or lack thereof) of 13 most
423 significantly differentially expressed miRNAs in each of the gene sets.

424 Additionally, a set of 19 most differentially expressed miRNAs was provided for the
425 MIENTURNET (17) online tool. After predicting the gene targets using mirTarBase 8.0 (20)
426 the resulting gene sets were used in an enrichment analysis using the Reactome database
427 (21) with 2 intersections.

428 Data from the FANTOM5 Project - promoter level mammalian expression atlas (18) were
429 used to determine the cellular origin of most differentially expressed miRNAs. Relative

430 abundance was calculated as a ratio of each candidate miRNA expression in a specific cell
431 type (tags per million) to the global expression level in all characterized cells.

432 **Statistics**

433 T Student's test was used for comparing normally distributed values between unpaired
434 observations, with Holm's p value correction for multiple comparisons. Paired data was
435 analyzed using paired T Student's test with Holm's correction where appropriate. Next-
436 generation sequencing derived data were analyzed using Wald test with Benjamini-
437 Hochberg FDR to correct for multiple comparisons. P values < .050 were considered
438 significant.

439 **Study approval**

440 Institutional Review Board of the Charité Universitätsmedizin Berlin approved this study
441 **EA1/079/06**. Written informed consent was acquired prior to participation in this study.

442 **Author contributions**

443 WF wrote the initial manuscript, analyzed the data and designed the model and the
444 computational framework. KP wrote the initial manuscript, conceived and performed the
445 experiments and analyzed the data. (WF and KP contributed equally to this work, names in
446 alphabetical order). MD acquired the clinical samples, verified the analytical methods,
447 performed the experiments and revised the manuscript critically. SDB contributed to the
448 design and implementation of the research, to the analysis of the results and revised the

449 manuscript critically. MB conceived the analytical approach, developed the theoretical
450 framework, supervised the findings of this work and wrote the manuscript. MW conceived
451 of the presented study, supervised the clinical aspect of the study, developed the
452 theoretical framework, aided in interpreting the results, and revised the manuscript
453 critically.

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462

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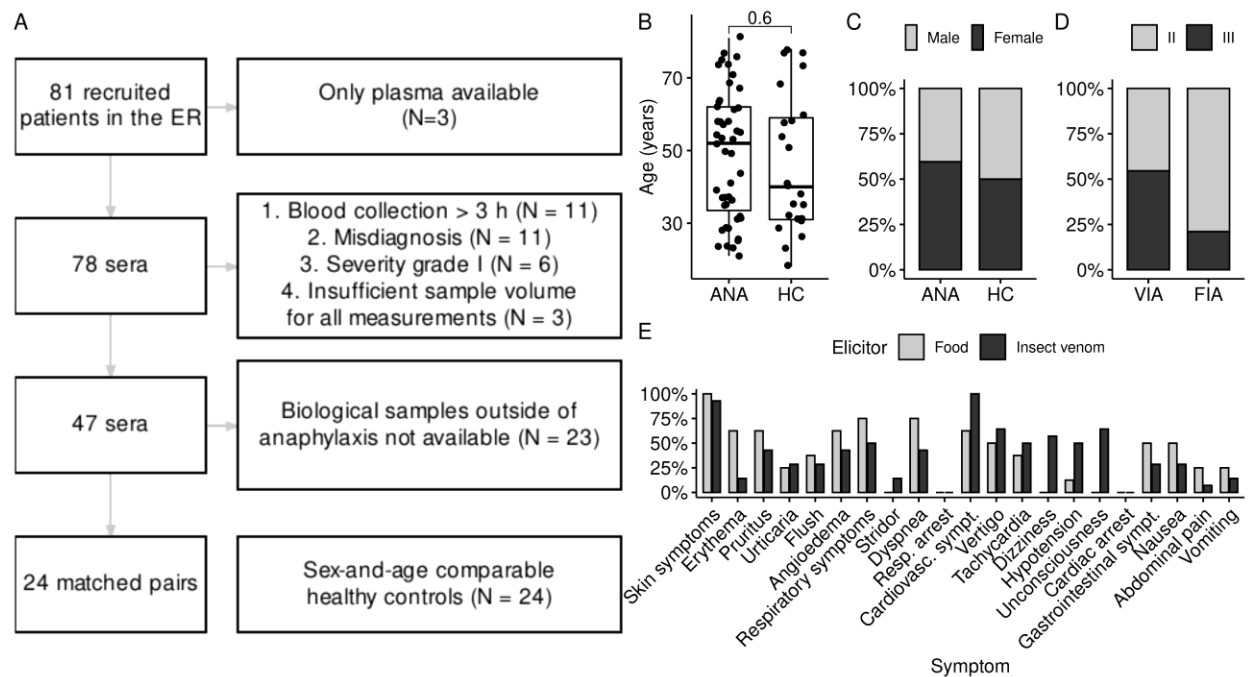
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596



598

599 **Figure 1: Studied cohort shows clinically relevant reactions.** A) Flowchart illustrating the
600 exclusion criteria of the study. B – C) Age and sex distribution in the studied cohort and their
601 corresponding controls (ANA - acute anaphylaxis, HC - healthy control group, two-tailed
602 Student's T test, $n = 71$). D) Distribution of elicitors and severity of anaphylaxis according to
603 Ring and Messmer (VIA – venom-induced anaphylaxis, FIA – food-induced anaphylaxis). E)
604 Frequency of symptoms in food and insect venom elicited anaphylaxis.

605

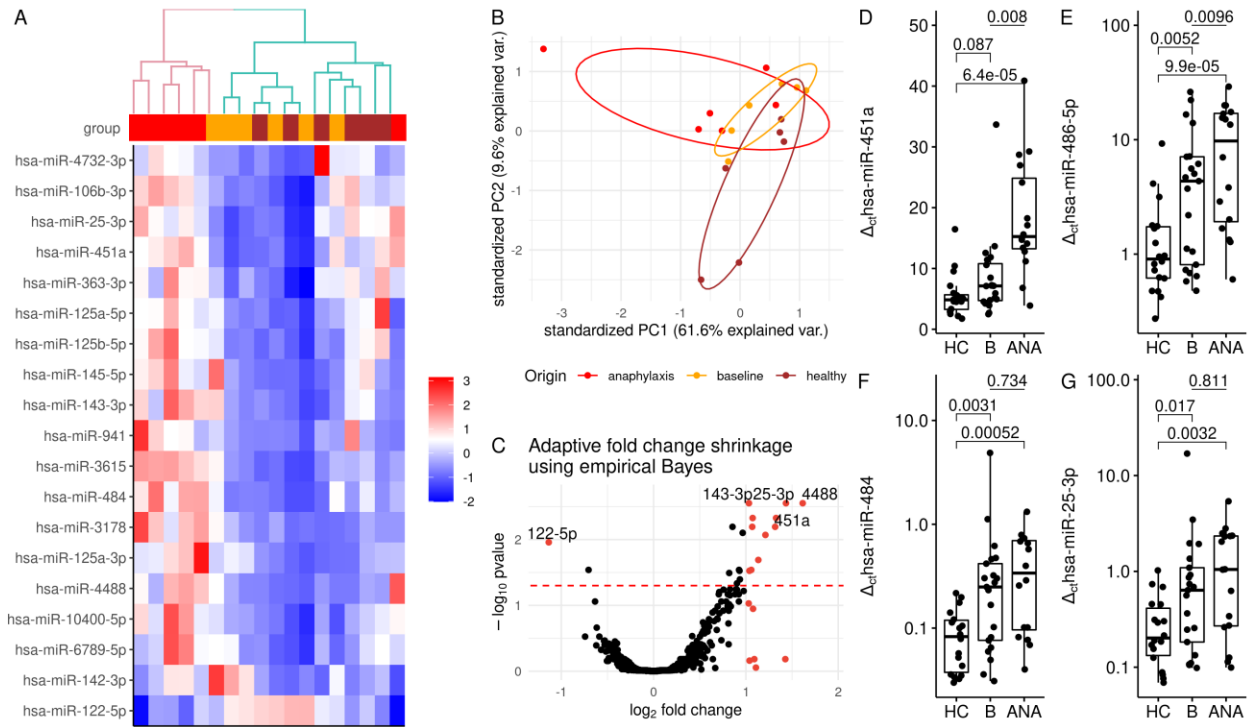


Figure 2: Screening and validation of miRNA candidate biomarkers show different expression in anaphylaxis when compared to healthy and baseline. A) Heatmap of 19 most differentially expressed miRNAs (using next generation sequencing) in 6 anaphylaxis samples (top row red) in comparison to the corresponding samples on baseline (top row yellow) applying unsupervised hierarchical clustering with euclidean distances. Additional 6 healthy control samples (top row brown) were provided for completeness. B) Principal component analysis of the sequenced cohort in corresponding colors (50 most differentially expressed miRNAs, data were scaled and centered). C) Volcano-plot illustrating differentially expressed miRNA upon anaphylaxis ($n = 6$) compared to baseline ($n = 6$). Fold-changes were adjusted using the 'ashr' algorithm. Red dots mark miRNAs with adjusted log-fold change > 1 and < -1 . Red dashed line indicates adjusted $p < 0.05$ (Wald test with Benjamini-Hochberg FDR, $n = 2656$ miRNAs per sample). D – G) Quantification of selected miRNAs in serum using real-time qPCR on anaphylaxis patients' sera in acute (ANA, $n = 16$), baseline (B, $n = 16$), and healthy controls (HC, $n = 20$). Comparisons between HC vs. B and HC vs. ANA - two tailed Student's T test for unpaired data with Holm's correction; comparisons between B vs. ANA - two tailed Student's T test for paired data.

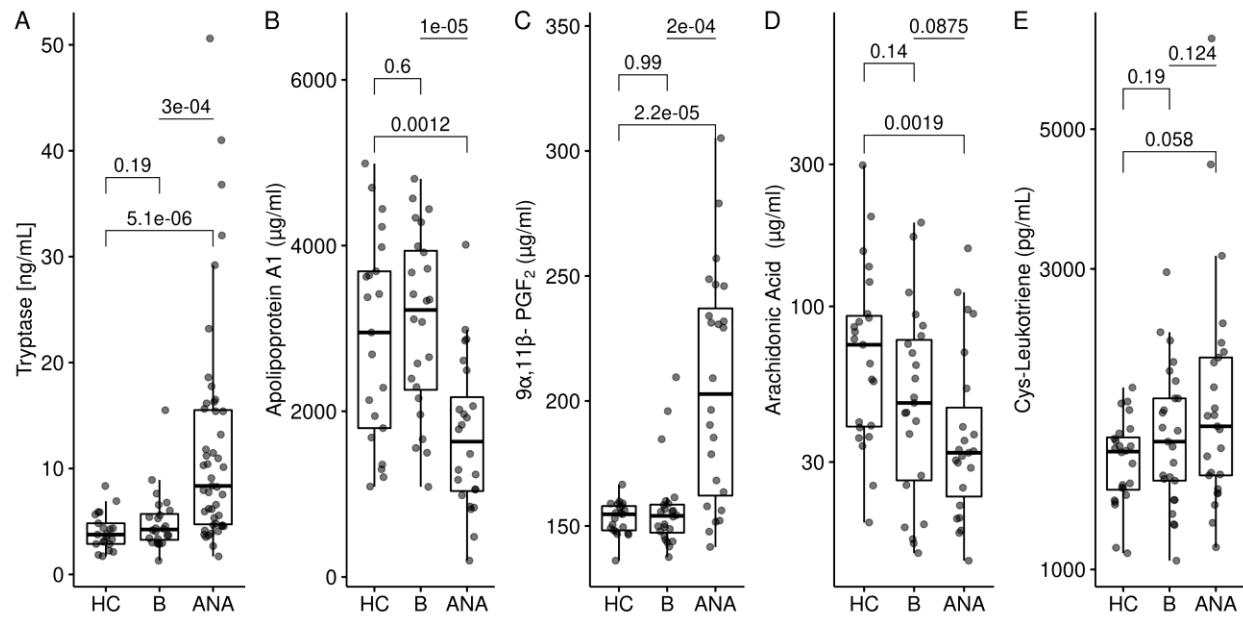


Figure 3: Protein and lipid candidate serum biomarkers differentiate between anaphylaxis and baseline. A) ImmunoCAP measurement of tryptase in serum; B – E) ELISA measurement of selected biomarkers in healthy controls (HC), baseline (B), and acute anaphylaxis (ANA). Comparisons between HC (n = 24) vs. B (n = 24) and HC vs. ANA (n = 24) - two tailed Student's T test for unpaired data with Holm's correction; comparisons between B vs. ANA - two tailed Student's T test for paired data.

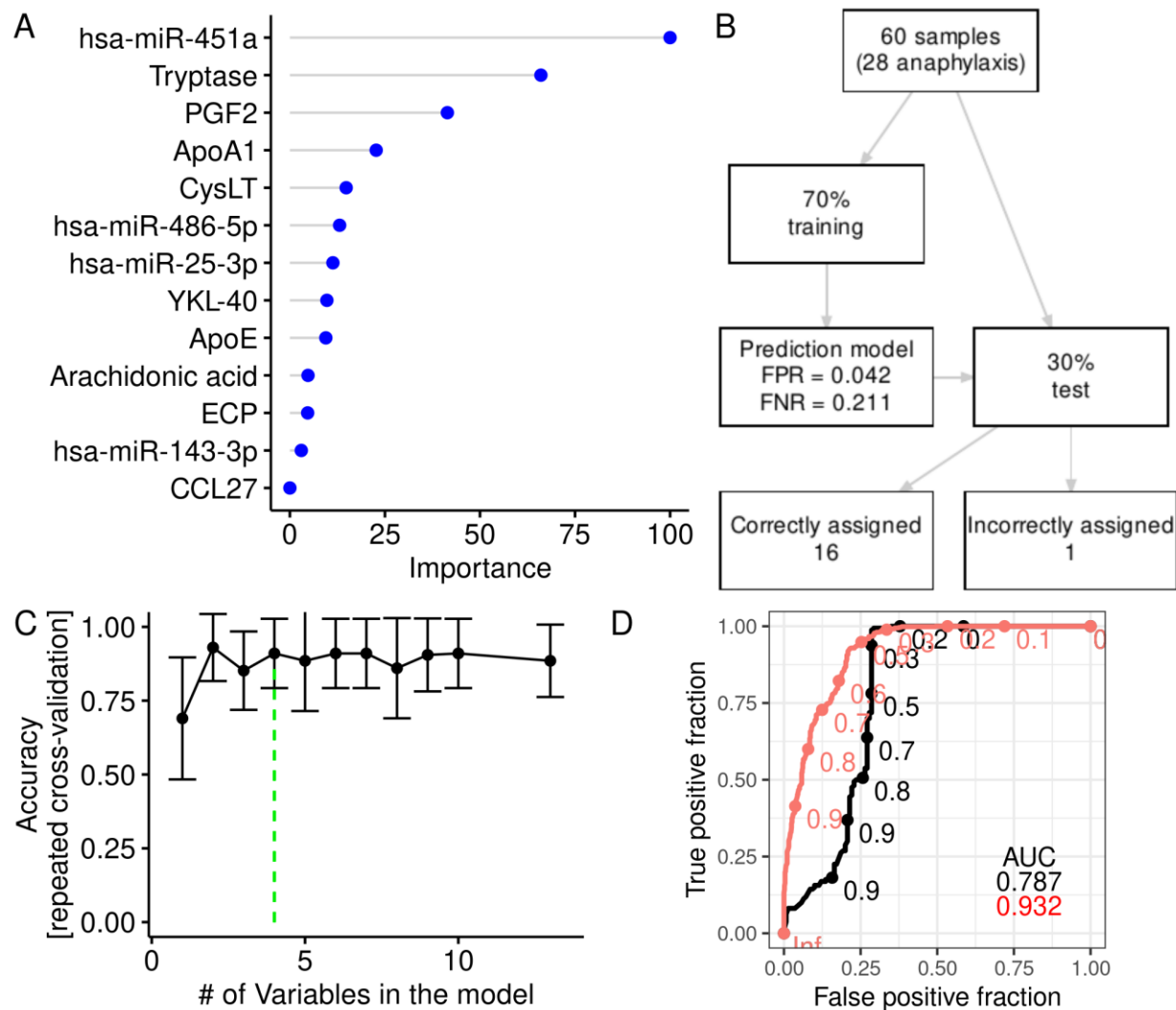
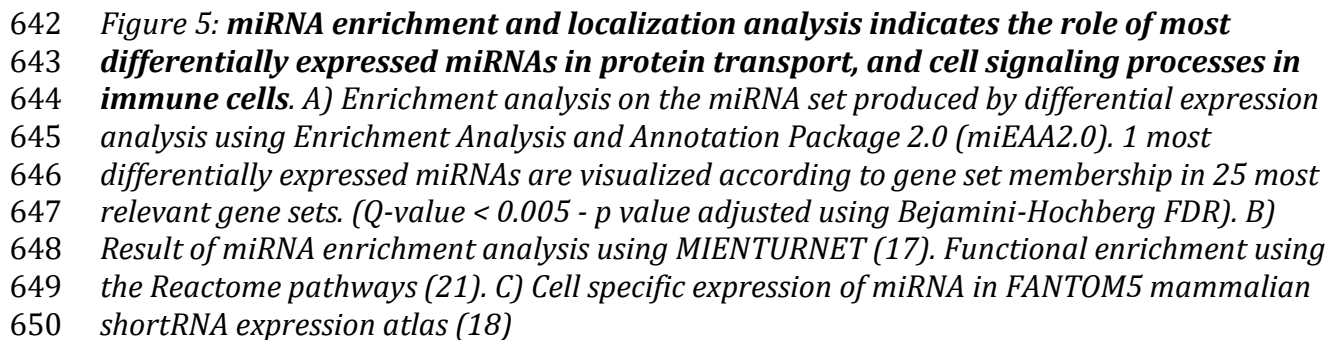


Figure 4: Serological biomarkers allow predicting an acute anaphylactic episode using random forest classification model. A) Feature importance score using random forest algorithm with all potential biomarkers of anaphylaxis. B) Summary of sample selection for the algorithm training and test sets. C) Automatic selection of best features out of the 18 initial ones reported in panel A using recursive feature elimination (RFE) algorithm (15). D) Receiver Operating Characteristic (ROC) in a random forest model with 4 top predictors (hsa-miR-451a, tryptase, PGF2, apolipoprotein A1 - red curve) compared to a model including tryptase, apolipoprotein A1 and PGF2 - black curve.



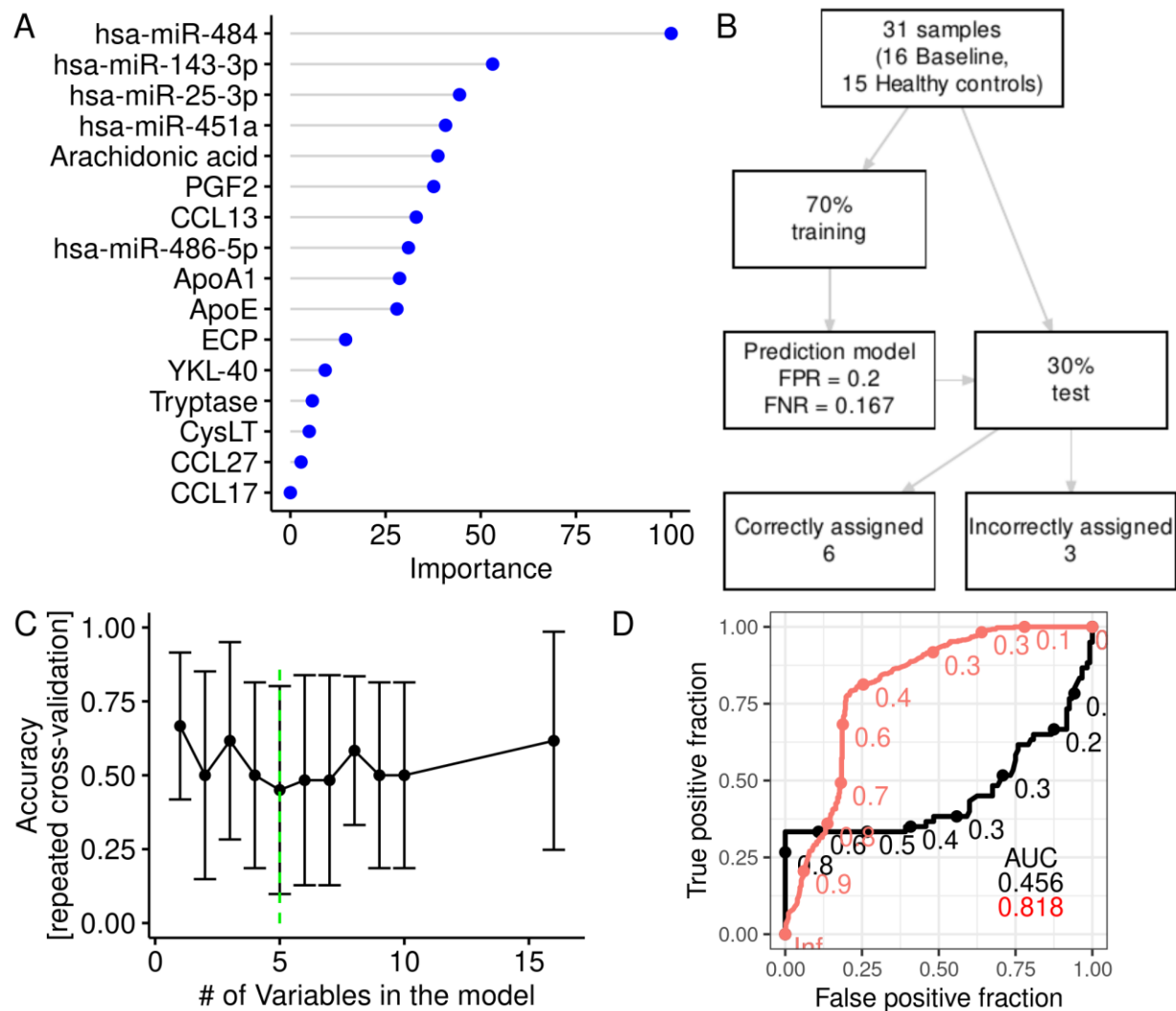


Figure 6: Serological biomarkers show potential to predict patients at risk of anaphylaxis. A) Feature importance score using random forest algorithm with all potential biomarkers. B) Flowchart for the modeling procedure including model evaluation using repeated cross-validation. FPR - False Positive Rate, FNR - False Negative Rate C) Automatic selection of best features out of the 17 initial ones reported in panel A using recursive feature elimination algorithm. Receiver Operating Characteristic area under the curve (AUC) in a random forest model with 5 predictors (hsa-miR-484, hsa-miR-25-3p, hsa-miR-451a, apolipoprotein E, YKL-40 - red curve) compared to a model including tryptase, apolipoprotein A1 and PGF2 - black curve.

